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RADIOIRON STUDIES IN A CASE OF HEMOCHROMATOSIS

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INTRODUCTION

HEMOCHROMATOSIS, a disease associated with disordered iron metabolism, lends itself to study with tracer doses of radioiron. In this paper a young adult European man, suffering from the disease and showing myocardial failure, sexual hypoplasia, and hepatic enlargement, is presented. At the suggestion of Drs. J. and T. Gillman a series of radioiron studies were carried out in this case and the results are recorded below.

CASE HISTORY OF PATIENT D. M., AGED 21 YEARS

The patient, who was a prison warder, presented initially on Aug. 8, 1948, with a history of several bouts of "palpitations" on the day prior to admission to the hospital. Previous exercise tolerance was good and there was nothing of significance in the former history.

On physical examination there was a complete absence of facial and axillary hair and the pubic hair had a female distribution. There was no abnormal skin pigmentation. There was no breathlessness at rest. Lying at an angle of 45 degrees the patient had pulsatile neck veins 7 to 8 cm. above the angle of Louis. The pulse rate was 84 per minute and was irregular because of extrasystoles. The blood pressure was 90/60 mm. Hg. Clinically the heart was normal in size. On auscultation, a gallop was heard at the apex. The liver was felt two fingers below the costal margin. The edge was very firm, smooth, and nontender. The size of the penis was within normal limits but the testicles were atrophic.

Laboratory Data.—

Complete blood count: Hemoglobin: 14.5 Gm. per cent. Color index: 0.91. Erythrocytes (per cubic millimeter): 4,920,000. Leucocytes (per cubic millimeter): 11,400. Neutrophils: 68 per cent. Monocytes: 4.5 per cent. Lymphocytes: 24.5 per cent. Eosinophils: 3.0 per cent. Basophils: 0. Sedimentation rate: slight increase. *Antifibrinolysin titer:* ++++ positive. *Modified Ide test for syphilis:* negative. *Urinalysis:* nil. *Liver function:* Thymol turbidity: 3.5 units. Thymol flocculation: + positive. Takata Ara (Ucko's modification): negative. Icteric index: 8 units. Alkaline phosphatase: 20 King-Armstrong units. Blood proteins: 4.99 Gm. per cent albumin, 3.19 Gm. per cent globulin. Prothrombin index: 63 per cent. *X-ray of chest:* heart and lungs normal. *Electrocardiograms:* Constant low amplitude in Leads I-III. PR and QRS times, normal. Chest Leads CR I-VI: large inverted T waves in positions I-IV. Ventricular multifocal extrasystoles.

While in the hospital the patient was asymptomatic. However, in view of the persistent hepatic enlargement, a liver biopsy was carried out by Dr. T. Gillman after a short course of vitamin K therapy. Microscopic section showed the presence of hemochromatosis with heavy iron deposits and an early portal cirrhosis (Fig. 1). The diagnosis of hemochromatosis focused more attention on the patient's endocrine disorders and further tests were carried out.

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Additional Laboratory Data.—

SUGAR TOLERANCE CURVE (AFTER 50 GM. GLUCOSE ORALLY)

DATE	FASTING	30 MIN.	60 MIN.	90 MIN.	120 MIN.
10/28/48	100 mg. %	200 mg. %	220 mg. %	155 mg. %	135 mg. %
3/10/49	90 mg. %	165 mg. %	135 mg. %	----------	95 mg. %

Duodenal intubation: normal quantities of ferments. Fats in stools: 83 per cent of twenty-four-hour specimen water. Of solids, 48 per cent fats, 10 per cent being unsplit. Serum and urinary diastase: normal. Blood chlorides: normal. Kepler-Robinson-Power test: negative. 17-Ketosteroids in urine: very low. Exact reading unobtainable because of interfering pigments. Blood cholesterol: normal. Basal metabolic rates: –35 per cent, –17 per cent, –25 per cent.

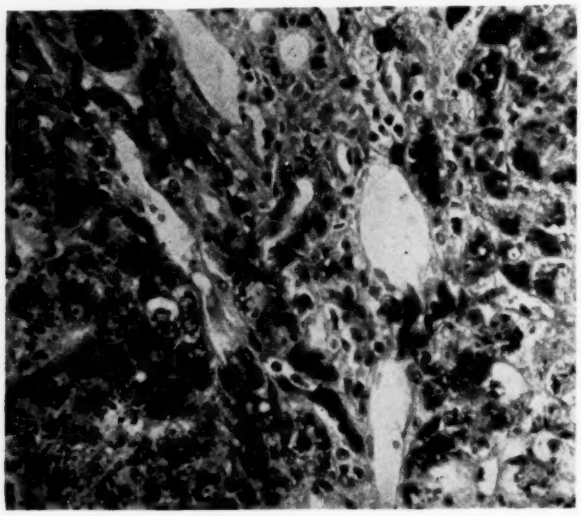

Fig. 1.—Microscopic section of liver showing heavy deposits of iron-containing pigment. (Stained with Prussian blue. ×320.)

The patient was well enough to be discharged on Dec. 21, 1948. He was readmitted on Jan. 1, 1949, with a week's history of attacks of "palpitations." On examination it was found that he was having paroxysmal bursts of tachycardia which, on electrocardiography, were found to be ventricular. These attacks responded to quinidine therapy and he was well enough to be discharged on Feb. 5, 1949. During the week of March 16-23, 1949, the radioiron studies, recorded below, were conducted.

On May 7, 1949, the patient was again readmitted with a two-week history of increasing breathlessness on exertion and of "palpitations." On examination it was found that he was in congestive cardiac failure. He was treated by bed rest, a salt-free diet, and digitalis. After a week all signs of congestive cardiac failure had disappeared and he was discharged. When the patient was last seen alive on July 3, 1949, he was still able to carry out light duties. However, on Aug. 8, 1949, he collapsed in the street and was dead before admission to the hospital.

A post-mortem examination was carried out by a pathologist unaware of the patient's antecedent history. The diagnosis of hemochromatosis was confirmed, but no special mention was made of the heart, except that there was some generalized enlargement (weight, 16 oz.).

MATERIAL AND METHODS

It was decided that oral administration of radioiron would yield the best information as to the fate of dietary iron, and for this purpose ferrous sulfate was obtained from Tracerlab, U.S.A. The activity as given by the suppliers on the date of dispatch was 0.215 mc. Fe^{59} and 0.035 mc. Fe^{55} in 15 ml. solution, the total solution containing 256 mg. Fe, i.e., 17 mg per milliliter. The amount of activity administered was determined on the basis that in the first few days the whole of the radioiron would be distributed in the alimentary tract, i.e., in about 4 kg. of tissue. On this basis it was calculated that 53 μc would deliver less than tolerance (0.1 r per day). The dose actually given was 2 ml. of solution, i.e., 28 μc of Fe^{59} on the day of administration, according to the Tracerlab standardization. This, however, did not agree with measurements in our own laboratory. An absolute calibration of the radioiron, using the disintegration scheme of Deutsch and co-workers,[4] gave the gamma ray activity as only 0.55 of the Tracerlab estimate. It seems reasonable to suppose that the radiation received by the patient was not greater than the accepted maximum permissible dose.

No measures were taken to alter the patient's diet in any way before the radioiron was given, and it seemed a fair assumption that the uptake by the patient of iron, and its metabolism thereafter, would give a picture of the fate of an equal quantity (i.e., 34 mg.) of iron in the diet.

In order to trace the course of the iron the following measurements were made.

(a) Assays of all radioiron excreted in the feces and urine over the first seven days after administration of the iron.

(b) In vivo measurements over various organs over the first seven days, and at certain intervals thereafter.

(c) Measurement over the same organs of a cadaver into which a model liver, containing radioiron, had been placed.

(d) Assays of radioiron in blood samples taken first at hourly then two hourly intervals for two days, and at suitable intervals thereafter.

(e) Assay of radioiron in liver biopsy material obtained three days after administration of the iron.

In Vivo Measurements and Cadaver Measurements.—It was found that the efficiency of an end window counter was higher for gamma ray counts from iron[59] than any gamma ray counter available to us. A General Electric Company type GM2 counter (copper window) was used for the in vivo measurements. It was assumed that radiations from Fe^{55} (energy 5.9 ekv) could play no part in the counts and the Fe^{59} half life, 47 days, was used in correcting results for decay.

Measurements on Excreta.—

(1) *Feces:* In the earliest stages where large amounts of radioiron were excreted in the stools, aliquots were digested and assayed by means of a liquid sampling counter of the Veall[13] type. The efficiency of the same counter was measured for a solution made up from the original stock, so that quantities eliminated were determined in terms of the dose administered to the patient. In later stages, where the quantities excreted were smaller, the plating technique, described below, was used.

(2) *Urine:* It was found that the count due to iron was not sufficiently high to allow use of a liquid counter. The iron from the urine was therefore plated.

Plating Technique.—The plating technique used was essentially that of Vosburgh, Flexner, and Cowie.[14]

Several of the first plates from urine and feces specimens were found to be covered with a layer of insoluble phosphates. The iron from these specimens was therefore precipitated with cupferron before the plating was carried out.

Measurement of Plated Samples: Standards.—The copper discs onto which the iron was plated were counted by means of a Victoreen end window counter (window thickness 3.4 mg. per square centimeter). It was not known how efficient the counting system was for radiations from Fe^{59} or from Fe^{55}, nor were

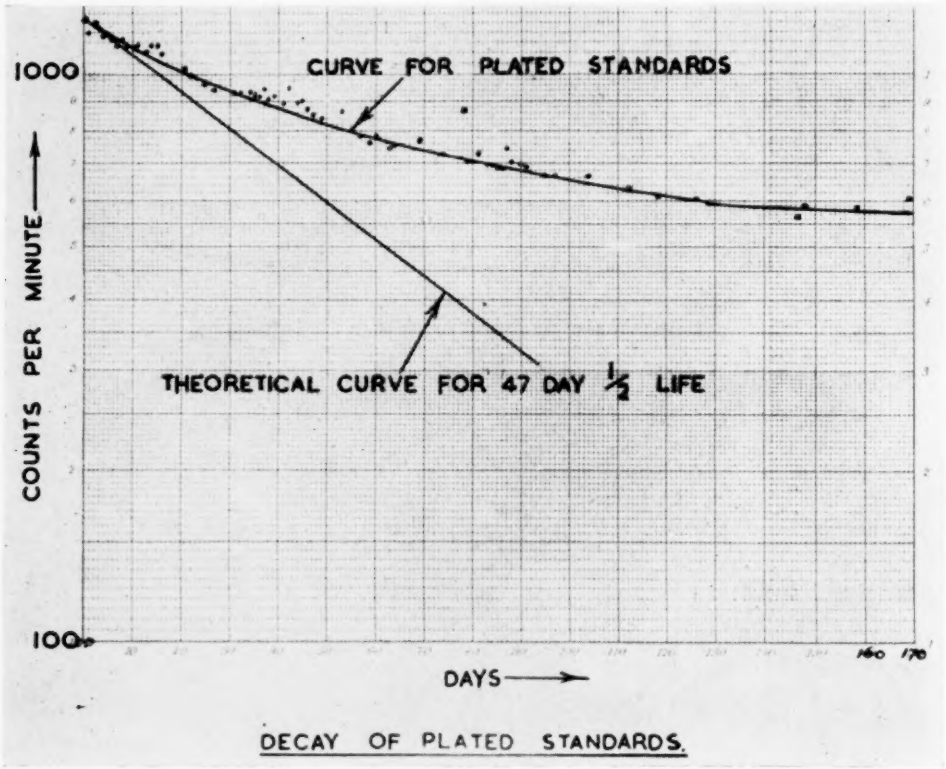

Fig. 2.—Curve showing decay of plated standards.

the absolute initial activities of these two iron isotopes considered to be sufficiently well known to be able to interpret the counts on plated samples in terms of disintegration rate. A measured aliquot, viz. 1/6,000 of the dose given to the patient, was therefore plated onto each of four copper discs, and these four standards were used as a basis of comparison. Fig. 2 shows the decay in these standards over eight months. It can be seen that the decay rate is much less than for iron[59] (47-day half life). A considerable and proportionately increasing number of counts appears to be due to the presence of iron.[55] All platings were therefore corrected in terms of counts made on the standards on the same day, and all results are given in terms of the total amount of radioiron given to the patient (or the control).

Control.—One healthy, normal control was used. He was given the same volume of radioiron solution as the patient. Measurements were carried out on the control similar to those made on the patient. After two days the control appeared to have eliminated practically all the radioiron given and no sign of radioiron was found in any of the in vivo measurements except for a very low net count over the liver.

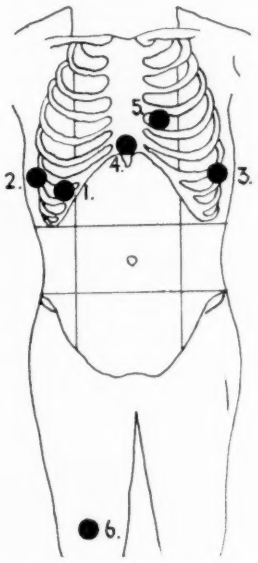

Fig. 3.—Sites over which in vivo and cadaver measurements were taken.

Cadaver.—It was soon established that the highest activity was concentrated in the liver of the patient. At the same time, when the Geiger-Müller counter was placed over other organs, counts considerably above background were obtained. The conditions where radioactive material is spread through a large organ of the body are not easy to imitate in vitro, and it was difficult to say how much of the count found over other organs was due to scatter from the radioiron in the liver. A model liver was therefore made up of "printer's roller composition" in which about 7 μc of radioiron had been dispersed. A cadaver was chosen of about the same physical dimensions as the patient. The model liver was larger than that removed from the cadaver and when it was put into position it approximated fairly well the physical conditions of the patient, whose liver was enlarged. Counts were made over various sites as indicated in Fig. 3.

RESULTS

(a) *Excreta.*—See Tables I and II.

The errors in determining the amounts of radioiron in the feces are probably large (±30 per cent) as small aliquots had to be taken, and with the crude mixing methods at our disposal we were not able to attain satisfactory homogeneity.

TABLE I. FRACTION OF ADMINISTERED DOSE IN TOTAL URINE

DAYS AFTER ADMINISTRATION	PATIENT (FRACTION, $\times 10^{-6}$)	CONTROL (FRACTION, $\times 10^{-6}$)
1st 24 hr.	317	46.1
2nd 24 hr.	28.8	3.95
3rd 24 hr.	12.2	6.2
4th 24 hr.	11.8	7.6
5th 24 hr.	4.4	12.6
6th 24 hr.	3.2	2.8
7th 24 hr.	3.1	Not measurable
8th 24 hr.	Not measurable	

TABLE II. FRACTION OF ADMINISTERED DOSE IN TOTAL FECES

STOOL PASSED	METHOD OF MEASUREMENT	FRACTION	STOOL PASSED	METHOD OF MEASUREMENT	FRACTION
20 hr. after administration	Liquid counter	0.38	1st day	Liquid counter	0.012
3 days	Plating	2.6×10^{-3}	2nd day	Liquid counter	0.56
5 days	Plating	0.0063×10^{-3}	3rd day	Liquid counter	0.29
6 days	Plating	0.035×10^{-3}	4th day	Plating	0.66×10^{-3}
			5th day	Plating	0.52×10^{-3}
Total: About 38%			Total: About 86%		

(b) *Blood.*—

(i) *Patient:* The results are best presented graphically (Fig. 4). The percentage uptake in the total blood is calculated on the assumption that the total blood volume was 4,500 milliliters. More accurate figures are not available.

A logarithmic time scale is used in order to bring out the initial peak which occurred at about three hours after the administration of the iron. It is presumed that this peak represents the initial uptake of iron in the plasma. The drop in radioiron in the blood and subsequent rise shows how the iron was removed from the plasma, stored, and gradually built into the hemoglobin until after three months something of the order of 4 per cent of the total administered iron was found in the red blood cells. In the sample taken on the sixth day (10 c.c.) the cells were centrifuged off, and all the radioiron was found to be in the cells—there was none in the plasma.

(ii) *Control:* No measurable amount of radioiron was found in any of the blood specimens taken until the third day. On the third and fourth days after measurement something of the order of 0.1 per cent of the administered dose per 4,500 ml. of blood was found. This was barely measureable (1/1,500 of 0.1 per cent in 3 ml. blood). No subsequent blood specimens were taken.

(c) *Liver Biopsy.*—The biopsy was performed on Patient D. M. three days after the administration of radioiron. Total dry weight of material received = 3 milligrams. The sample when plated gave a net count of 11 cpm, the standards on the same day counting 1,160 cpm. Thus 5.2×10^{-4} of the total dose was found in the liver, per gram of dry weight, or about 1.0×10^{-4} of the total dose per gram of fresh weight. It is of interest to compare this with the

result of the assay on the liver made after postmortem (see section e), when the count was 1,830 cpm per gram dry weight, the standards giving at that time a count of 543 cpm. The fraction of total dose per gram dry weight of tissue was therefore 5.3×10^{-4}. In view of the large probable error involved both in weighing and in working with only 3 mg. of material, the agreement is satisfactory—but the accuracy of the measurements is not sufficient to warrant any inference being drawn as to increase or decrease with time of the amount of radioiron in the liver. The in vivo measurements do in fact point to a slight decrease.

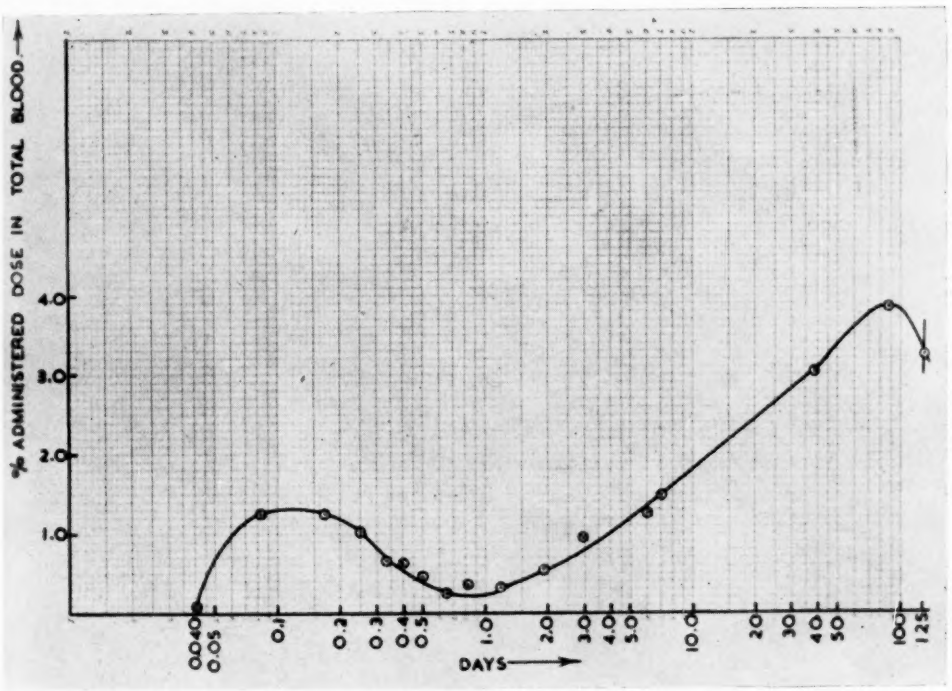

Fig. 4.—Uptake of radioiron in the blood of hemochromatosis patient.

Unfortunately, with no knowledge of the total liver weight it is not possible to say what total amount of radioiron was stored in the liver. If one could assume the liver to have been of normal weight, viz. about 1.6 kilograms, one could say that some 15 to 20 per cent of the administered dose had been stored in the liver. It is known that the patient's liver was in fact considerably enlarged: the percentage of administered radioiron stored in the liver might therefore easily have been as high as 30 per cent.

(d) *In Vivo Measurements.*—Many measurements were made on both patient and control over a total of twelve sites on the body. Two exemplary curves are given (Fig. 5), the time scale again being logarithmic. The readings have been corrected for the decay of Fe^{59} (47-day half life). The sites used in Fig. 5 correspond to sites *1* and *2* in Fig. 3. It can be seen that the storage of radioiron in the liver commenced very soon after the iron was given, and that

there was some decrease over the period 5 days to 125 days. The high counts over the heart over the first two days can probably be attributed to the presence of radioiron in the gastrointestinal tract. In the period 5 to 125 days there appears to have been some increase in the amount of radioiron present in the heart.

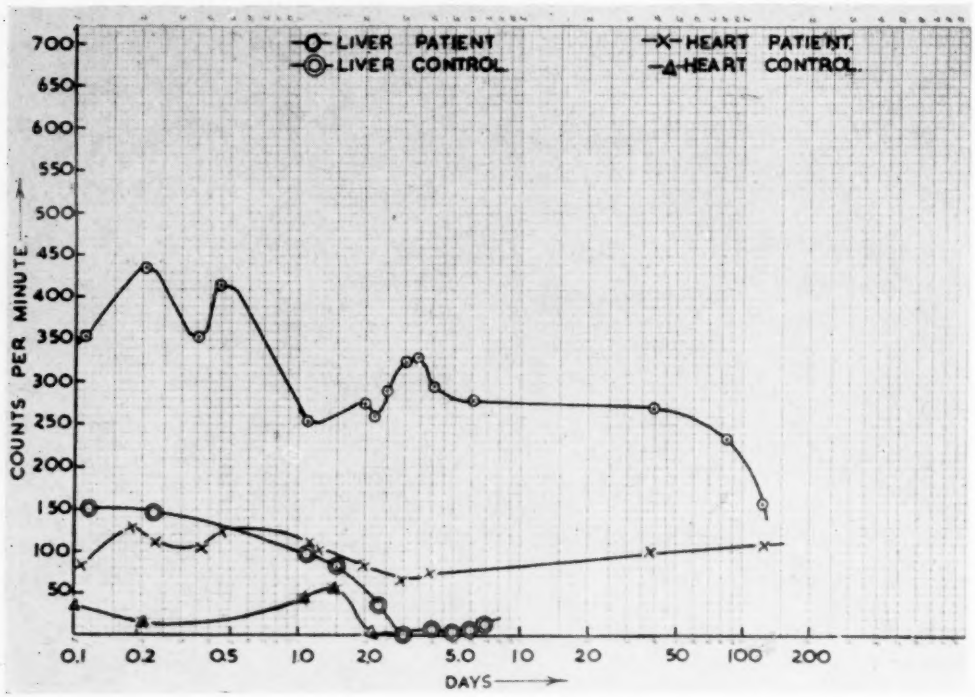

Fig. 5.—Counts per minute over mid-heart and mid-liver. Patient and control.

The control showed no count over any site, after elimination of a large fraction of the radioiron, except for a very low count over the liver.

The patient died suddenly on Aug. 8, 1949. Unfortunately the postmortem was carried out by an operator who was not aware of the special interest of the case. The weights of organs were not ascertained nor was it possible later to obtain certain organs for assay of radioiron. In view of the interest in the extent of iron deposition in the heart in hemochromatosis it was most unfortunate that we were not able to examine this organ for radioiron. Such organs as were recoverable were assayed. Some organs were assayed for total iron and copper.

(e) *Measurements on Organs Obtained From Postmortem.*—See Table III.

(f) *Measurements on Cadaver.*—Table IV shows the counts for both the patient and the cadaver over various sites, given as fractions of the count over Site 1, the site of maximum counting rate. (The counts were taken on the patient six days after the administration of the radioiron.)

TABLE III

TISSUE	CPM/GM. DRY WT.	FRACTION OF DOSE PER GM. DRY WT. $\times 10^{-4}$	% IRON (STABLE) FOUND IN ORGAN
Liver	1,830	5.32	0.726
Pancreatic lymph glands	860	2.64	0.371
Kidney	730	2.24	–
Mesenteric lymph glands	466	1.43	0.080
Pancreas	162	0.496	0.313
Spleen	118	0.362	–
Duodenum	81	0.249	0.0767
Pyloric end of stomach	41	0.126	–
Mass of mesentery lymph glands	17	0.052	–

TABLE IV

SITE	CADAVER (RATIO, CPM/CPM OVER SITE 1)	PATIENT (RATIO, CPM/CPM OVER SITE 1)
1	1	1
2	0.92	0.96
3	0.19	0.24
4	0.53	0.45
5	0.19	0.51
6	0	0.02

DISCUSSION

The feature of this study was the large percentage of 34 mg. radioiron taken up by the patient and retained in the body. Although Hahn[8] has shown that only a small percentage uptake can normally be expected in these circumstances, 1.4 per cent of the administered dose was circulating in the blood after six days and this figure gradually rose to a maximum of 4 per cent after 100 days. In addition, a large proportion of the radioiron was deposited in the liver. On excretion figures the patient retained more than five times as much as the control, but the in vivo measurements indicate that the true ratio was far greater than this. In contradistinction to the control, practically no radioiron was voided by the patient after the first twenty-four hours.

Our results for absorption as well as utilization of iron in hemochromatosis are in striking contrast with those of Balfour and co-workers[1] who fed 60 mg. of labelled iron to a 49-year-old hemochromatotic patient. The highest amount of circulating radioiron up to six days was found to be 0.012 per cent of the administered dose as against 1.4 per cent in our patient. No measurements other than blood uptake were made by these authors. The fact that our patient died from hemochromatosis at the early age of 21 years suggests that the disorder in iron metabolism was much more severe in his case.

Finch and associates,[5] who studied the utilization of *intravenous* radioiron, found that in three hemochromatosis cases the iron utilization in the blood was depressed considerably below normal, viz. between 10 and 20 per cent utilization after sixteen days, as against roughly 70 per cent for normal subjects. The figure obtained in the present study of 4 per cent utilization after 100 days must, however, be regarded as greater than normal, if we compare it with (a) the very low utilization of the control, (b) the utilization by a normal subject in the study of Balfour and co-workers,[1] and (c) the results of Darby

and co-workers[3] who fed 2 to 3 mg. of labelled iron to children between the ages of 7 and 9 and found a mean uptake of 13.5 per cent after ten to fifteen days. A percentage uptake of only one-tenth this figure, or less, could legitimately be expected from the feeding of fifteen times as much iron to a normal adult.

The uptake of iron in the liver was very marked: at least 15 per cent and possibly more than 30 per cent of the administered radioiron was found there after five months. Accepting the conditions after six days as stable, we see from the in vivo measurements that the storage of iron in the liver at that time may have been as high as 60 per cent.

Measurements over the heart were almost three times as high as could be expected if the counts were due to scattered radiation from the iron in the liver. It is evident that in this case significant deposition took place in the heart after a relatively short time. Records of twenty-two cases of hemochromatosis, showing myocardial failure, revealed deposits of iron in the heart muscle in all but one case (Bothwell and associates[2]). However there was no correlation between the amount of iron deposition and the degree of myocardial failure.

Sheldon[10] in his monograph concluded that if there is increased iron absorption in hemochromatosis, then it must be very slight and be associated with the slow accumulation of iron in the body over many years. If very high intestinal absorption can occur in the disease, as is suggested by our results, then it becomes important to study further the factors which may modify such absorption. Increased iron absorption has already been produced in cats after ligation of the pancreatic ducts[11, 12] and in rats fed on an iron-supplemented corn-grit diet.[9] This increased absorption can be partially prevented in the cats by the addition of Haliverol to the diet,[12] and in the rats by the addition of phosphate.[9] The Gillmans[6, 7] have shown heavy accumulations of iron in the livers of many Bantu pellagrins, with microscopic features, in the later stages, indistinguishable from hemochromatosis. The deficient Bantu diet of maize meal porridge may contain 100 to 150 mg. iron per day, the iron being derived from cooking pots.[13]

The reason for the increased iron absorption in hemochromatosis is not clear. The experimental evidence suggests that pancreatic damage may be an initiating factor. It is a matter for conjecture whether the addition of either Haliverol or phosphate to hemochromatotic patients' diets may help to arrest the course of the disease. Such a possibility invites studies with tracer doses of radioiron.

SUMMARY

Radioactive iron studies have shown that of 34 mg. (total) of iron taken orally in one dose by a hemochromatotic patient, with myocardial failure, sexual hypoplasia, and hepatic enlargement, about 60 per cent was retained. Most of it was stored in the liver; some was deposited in the heart and varying amounts were found to be present in all the organs examined after postmortem. The up-

take of radioiron in the blood could be clearly followed. The maximum uptake was about three months after the dose was administered and was about 4 per cent.

We wish to acknowledge our indebtedness to Professor G. A. Elliott, under whose care the patient was, for permission to publish this case and for his unfailing help and encouragement in the preparation of this paper. It has also been our privilege to discuss many aspects of the case with Drs. J. and T. Gillman whose researches have helped to throw new light on a very obscure condition.

Thanks are also due Drs. B. van Lingen, I. Welsh, and B. C. Eliis for their helpful cooperation, and Mr. G. Millner, Miss M. L., du Preez, Mr. L. P. de Valence, and Miss I. D. Reeler for many measurements.

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URINARY EXCRETION OF GALLIUM BY MAN AND ANIMALS

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INTRODUCTION

IN PREVIOUS studies it was demonstrated that parenterally administered gallium was rapidly deposited in osseous structures.[1, 2] It thus appeared that the element might prove useful in the study of bone metabolism. Studies with the radioactive isotope (Ga^{72}) both in the laboratory[3, 4] and clinically[5, 6] indicate the radioactive isotopes of gallium are of particular interest. Since urinary excretion was shown to be the chief means of clearance of this element,[7] more detailed studies of this phase of the metabolism of gallium were indicated.

The present report deals with (a) a chemical method for the determination of gallium in urine, (b) a study of the influence of dose and route of injection on the urinary excretion of gallium by the rabbit and the dog, and (c) a study of the gallium excretion and blood levels of patients receiving single doses of gallium citrate intravenously. The gallium was the carrier which accompanied Ga^{72} used in the study of persons having neoplastic lesions involving bone.

METHODS

The chemical procedure for the determination of gallium in urine is based on the finding that gallium may be quantitatively extracted from 6N HCL solutions by diethyl ether.[8] After separation of the gallium, it is converted to a 8-hydroxyquinoline complex in chloroform solution.[1, 9] In the range of 0.05 to 1.5 mg., the yellow color of the complex is measured in a photocolorimeter. In the range of 1 to 10 μg the fluorescence produced by ultraviolet light is used to estimate the gallium content of unknowns.

Analytic Procedure.—To 20 c.c. of freshly voided urine are added 10 drops of concentrated (38 per cent) HCl to dissolve all precipitates. Add 4 c.c. of 40 per cent hydroxylamine hydrochloride solution and 1 c.c. of 10 per cent sodium acetate solution. Let stand in a stoppered container for at least ten minutes. Add 20 c.c. of concentrated HCl, cool, and extract 10 c.c. of this mixture twice with 10 c.c. portions of acidified ethyl ether. The ether should have been previously shaken with twice its volume of 6N HCl.

The ether extracts are combined and extracted twice with 10 c.c. portions of distilled water. The gallium chloride passes into the aqueous layer.

To the aqueous gallium solution, add 2 c.c. of 40 per cent hydroxylamine hydrochloride and 1 c.c. of 10 per cent sodium acetate solution. Adjust the pH of this mixture to 2.5 to 2.8. The control of the pH at this point is of greatest importance to insure specificity. Add 2 c.c. of 5 per cent 8-hydroxyquinoline sulfate (previously adjusted to pH 2.6).

Extract the hydroxyquinoline complex with two 5 c.c. portions of redistilled chloroform. Adjust the final volume to 10 c.c. with a small amount of chloroform; cover with about 0.5 c.c. of the water phase. Let stand for at least fifteen minutes and compare with standards. In the range 0.05 to 1.0 mg., the yellow color is a measure of the gallium in the chloroform solution. In the range 2 to 10 μg, fluorescence may be used to estimate the gallium content in a suitable photofluorometer having an ultraviolet source.

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The opinions or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or endorsement of the Navy Department.

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*Commander, MSC, USN.

Radiochemical and gamma counting technique have been given previously.[4]

Intravenous injection of gallium citrate and Ga^{72} citrate was made by methods described previously.[4, 5]

Notes on Analytic Procedure.—

(a) Prepare hydroxyquinoline and hydroxylamine solutions fresh weekly and store under refrigeration.

(b) In the colorimetric procedure recovery at 0.005 mg. is 70 per cent of the added gallium. In the range 0.01 to 1.0 mg., recovery is 96 to 98 per cent of added Ga. Using the fluorometric technique, at 1 to 2 μg gallium recovery is 80 per cent of added standard amounts, while in the range of 3 to 10 μg recovery is essentially quantitative.

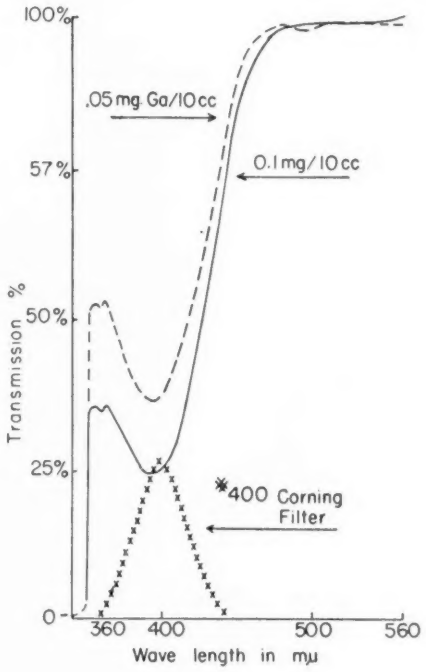

Fig. 1.

The specificity of this method depends on the reduction of iron by hydroxylamine in the presence of sodium acetate. In the ferrous state iron is not extracted by ether from 6N HCl. Such iron as may normally be carried into the final 8-hydroxyquinoline solution will offer no difficulties at a pH of 2.5 to 2.8. Dark color in the chloroform solution indicates the presence of excessive amounts of iron and a probable pH of 3 or more in the final extraction procedure. Low values in the fluorescent range will be obtained if the final chloroform extraction is at pH less than 2.5. Indium may interfere but has not been found in the urine samples studied. No normal constituents of animal or human urine have been noted in routine sampling which give fluorescence under the conditions of this procedure. High blanks can be expected if the conditions are not controlled, and particularly if the 8-hydroxyquinoline solution is not prepared weekly.

(c) Prepare standards of 5 gamma and 0.5 mg. content and carry through the entire extraction procedure. Use these solutions as reference standards since this method will correct for unavoidable losses through extraction and will quickly indicate spurious results due to contamination of reagents.

(d) In Fig. 1 are shown the color characteristics of the 8-hydroxyquinoline-gallium complex in chloroform, and the light transmission of the filter used. The fluorescence produced by ultraviolet light is a continuum in the yellow-green range. Filters available with most photofluorometers are satisfactory for this emission.

(e) In Fig. 2 are shown the curves indicating the range in which the light transmission or emission is a straight-line function of the concentration of gallium-quinolate in 10 c.c. of chloroform.

(f) The final chloroform extracts if covered with a small amount of the aqueous phase (reducing atmosphere) are stable for twenty-four hours or longer if stored at 5 to 10° C.

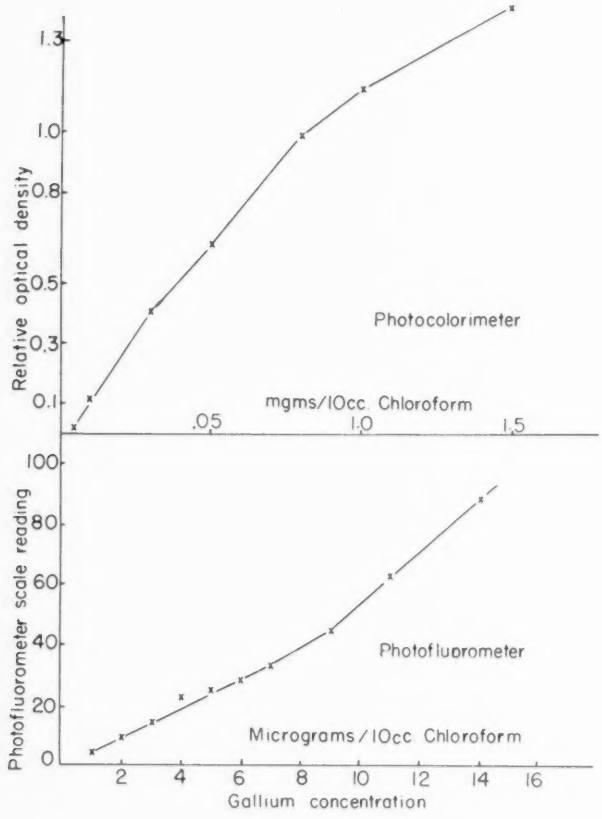

Fig. 2.

RESULTS

In Table I are shown the results of a study of the influence of the route of parenteral administration (intravenous or subcutaneous) on the urinary excretion of gallium by the rabbit. Also shown are the results of varying the dosage by subcutaneous injection. These results indicate that at a moderate (nontoxic) dose of gallium citrate, the rabbit retained a significantly greater amount of gallium if it was administered intravenously. When the subcutaneous dose was varied, it was found that the higher dose promoted a greater retention of gallium. At 45 mg. Ga per kilogram, the absolute amount

TABLE I. URINARY EXCRETION OF GALLIUM BY THE RABBIT

TEST	MG. GA INJECTED (AS CITRATE)	MG. GA EXCRETED IN: 0-24 HR.	24-48 HR.	48-72 HR.	72-96 HR.	% GA EXCRETED IN 48 HR.
		*Dosage: 5 mg. Ga/kg. subcutaneously**				
1	11.6	9.3	0.8	0.01	0.02	87.0
2	11.0	5.6	0.6	0.02	0.04	56.4
3	9.2	2.0	0.2	0.03	0.17	24.0
4	11.6	6.6	0.3	0.03	N.D.	59.4
5	10.2	9.0	0.7	0.05	0.08	95.1
6	11.0	9.3	0.3	0.01	0.04	94.1
		*Dosage: 5 mg. Ga/kg. intravenously**				
7	13.0	1.4	0.4	0.08	—	13.8
8	10.8	0.6	0.1	0.04	—	6.5
9	14.0	0.3	0.2	0.19	—	3.6
10	11.6	1.3	0.3	0.12	—	13.8
11	12.8	1.3	0.3	0.64	—	12.5
12	8.0	1.3	0.1	0.07	—	17.5
		Dosage: 45 mg. Ga/kg. subcutaneously†				
13	126	19.9	3.0	0.2	1.2	18.0
14	108	23.4	11.7	0.4	1.3	32.5
15	126	29.5	6.5	N.D.	Died	28.6
16	68	21.0	3.3	Died	—	35.7
17	126	18.8	19.4	3.0	1.3	30.3
18	104	31.2	2.3	0.4	0.8	32.2

*This dosage produces no significant effects or changes in the urine.

†This dosage is the LD_{50}, and produces within twenty-four hours marked changes in kidney function. Albuminuria and glucosuria are common.

(milligrams of Ga) retained approximated twenty times that retained at a dose level of 5 mg. Ga per kilogram (average of 6).

In Table II are shown the results of a study of the influence of route of administration and dosage on the urinary excretion of gallium by the dog (nine animals). The results indicate that intravenous injection of gallium citrate also induced greater retention. The influence of dosage is not so clear in the case of the dogs as in the case of the rabbits, probably because the two dose levels were close together (1 and 5 mg. per kilogram).

TABLE II. URINARY EXCRETION OF GALLIUM BY THE DOG

TEST	MG. GA INJECTED	MG. GA EXCRETED IN: 0-24 HR.	24-48 HR.	48-72 HR.	% GA EXCRETED IN 48 HR.
		Dosage: 5 mg. Ga/kg. subcutaneously			
1	50	35.6	None excreted	4.0	71.2
2	46	36.4	4.9	0.9	90.0
3	64	27.4	10.8	1.1	59.7
		Dosage: 5 mg. Ga/kg. intravenously†			
4	59	16.2	1.5	2.5	30.0
5	65	8.3	1.2	2.5	14.6
6	60	13.8	0.8	2.6	24.3
		Dosage: 1.0 mg. Ga/kg. intravenously			
7	11	None excreted	3.1	0.1	28.1
8	12	4.3	1.6	0.1	49.1
9	12	4.5	0.8	0.1	44.1

Gallium administered as the citrate, in solution containing 20 mg. Ga per cubic centimeter at pH 6.5.

†The LD_{50} for the dog is 10 to 15 mg. Ga per kilogram. The dose of 5 mg. Ga per kilogram produces minimal kidney changes which are often accompanied by albuminuria.

TABLE III. URINARY EXCRETION OF GALLIUM BY MAN

NUMBER OF MEN	DOSAGE (INTRAVENOUS) (MG. OF GA)	% GA EXCRETED IN 24 HR.
9	3.8-5.7	4-43
7	6.0-9.0	16-33
6	60-71	25-37

In Table III are shown the results of studies of the urinary excretion of gallium by patients (male and female) receiving intravenously doses of gallium citrate ranging from 3.8 to 71 mg. Ga. These were persons who had neoplastic conditions involving bone and received Ga^{72} citrate intravenously. These patients received the indicated quantities of carrier gallium, and the urine examinations reported in Table III were carried out by the chemical technique already described. These preliminary results indicate that the urinary excretion of gallium following intravenous administration of the citrate varies widely. These figures must be considered to be preliminary since it appears that the degree of pathologic bone involvement may influence the amount of Ga retained.[5] Additional studies of these relationships are under way.

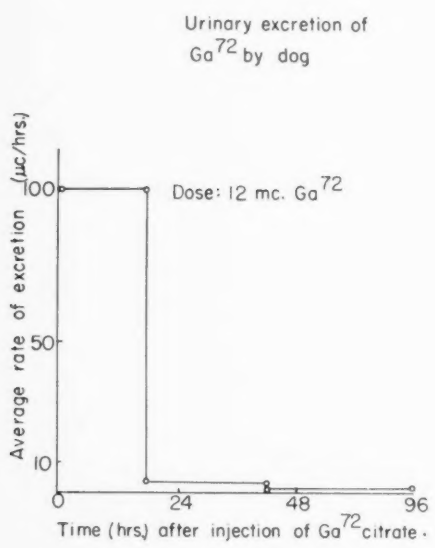

Fig. 3.

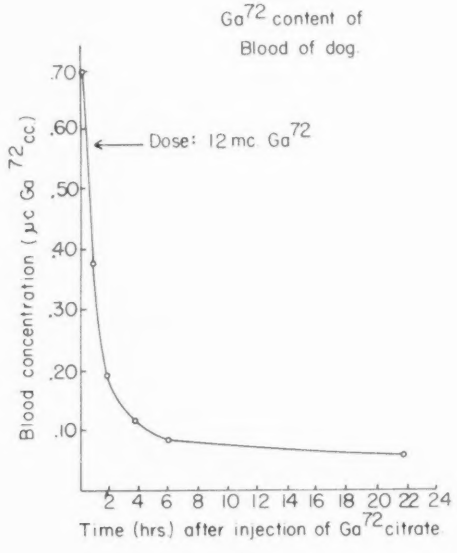

Fig. 4.

Studies of Radiogallium Excretion.—In Fig. 3 are shown typical results of studies on the urinary rate of excretion of Ga^{72} by the dog, following intravenous injection of gallium citrate (total, four dogs studied). These findings indicate early excretion, followed by negligible amounts. These findings are explained by the rapidly decreasing blood concentration of gallium (Ga^{72}) (Fig. 4) which reaches a low, relatively constant value within six hours after intravenous injection. The findings of the dog studies were essentially duplicated in the study of four patients receiving various doses of Ga^{72}.

Fig. 5 shows typical excretion curves, resulting from the study of a catheterized patient at two dosage levels, indicating marked early urinary excretion with decreasing amounts to twenty-four hours after injection. These findings are explained by the rapid fall in the Ga^{72} blood concentrations following intravenous injection (Fig. 6). From these results it appears that after four hours the blood-gallium level has reached an essentially constant value.

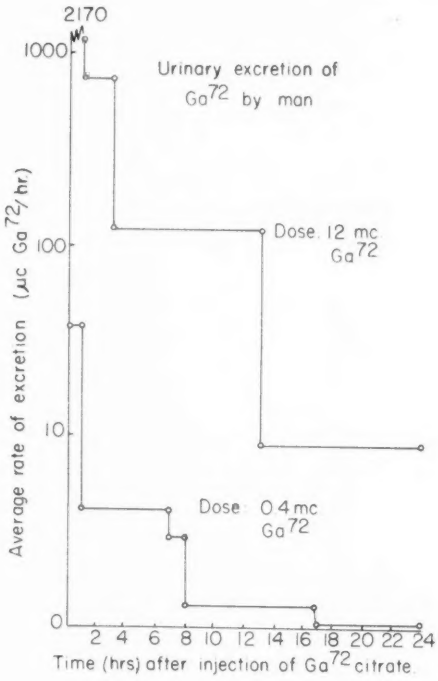

Fig. 5.

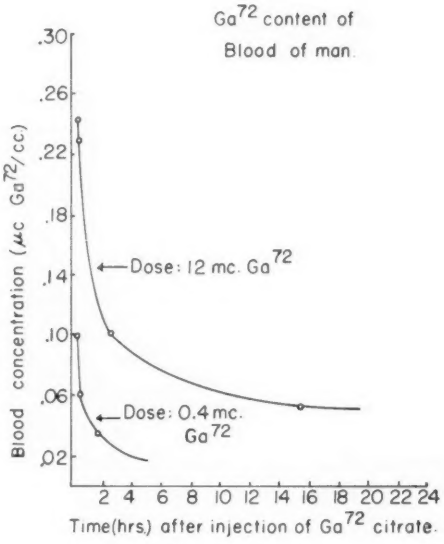

Fig. 6.

DISCUSSION

Laboratory findings indicated, in the case of rabbits and dogs, that urinary excretion is the chief means of clearance by these species. (Feces Ga/urine Ga = $\frac{1}{20}$ to $\frac{1}{50}$.) On the basis of these earlier findings, it was postulated that a study of the urinary excretion of gallium might prove a useful adjunct in the diagnostic studies utilizing radiogallium (Ga^{72}). However, the diuretic effect of intravenous citrate, differences in threshold of the kidneys, as well as conditions of polyuria may all contribute to a greater urinary clearance of gallium. These variables must be considered when evaluating the results of gallium excretion studies.

No explanation can now be offered for the increased retention of gallium following intravenous administration of gallium citrate. It would first appear that this would result from greater retention by the skeletal structure. Other

work in progress indicates that the uptake in bone is a function of the dose but not the route of administration. Thus it appears that intravenous injection of gallium induces greater retention by deposition in the soft tissues. The rapid disappearance of Ga citrate from the blood stream indicates that this compound may be a colloid, and the disappearance resulted from phagocytic action. To determine if gallium citrate (at pH 7.4) was a macro molecule, or colloidal in nature, solutions of the compound were placed in cellophane bags, with and without the presence of horse serum. In all cases the gallium rapidly dialized through the cellophane. Thus it appears that gallium is in a true solution. Slight ionization of the gallium citrate is indicated, since the usual gallium salts [$Ga(NO_3)_3$, $Ga\,Cl_3$] produce a curdlike precipitate with proteins.

SUMMARY AND CONCLUSIONS

A method is described for the separation of gallium from urine by ether extraction of 6N HCl solution. Colorimetric or fluorometric estimation of 8-hydroxyquinoline-gallium complex in chloroform is the basis for quantitative determination.

In the rabbit and dog, intravenous injection promotes greater retention of parenterally administered gallium citrate, with lessened urinary excretion.

The twenty-four hour urinary excretion of gallium by twenty-two patients varied widely.

Studies of the rate of urinary excretion of Ga^{72} in man and dog indicate that in both the initial excretion rate is high and reaches a low level in less than eighteen hours. Blood gallium levels fall rapidly after intravenous injection and approach low constant values within six hours.

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FURTHER STUDIES ON RECOVERY FROM RADIATION INJURY

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INTRODUCTION

IT HAS been reported by Jacobson and associates that the LD_{50} of whole body x-irradiation for intact young adult mice is circa 550 r and that only rarely will a mouse survive a dose above 800 r, whereas 40 per cent survive a dose of 1,025 r if the spleen is mobilized surgically and lead shielded during the exposure.[1] These reported mortality data represent the early experience with this technique; the more recent data are shown in Table I. These latter data indicate that with added experience with this technique fewer animals succumb and circa 77.7 per cent of mice exposed to 1,025 r (with spleen shielding) survive compared to circa 40 per cent survival in the early experiments; in fact, 55 per cent of spleen-shielded mice survive 1,100 r.

It was also reported that, in contrast to the same exposure without spleen shielding, no anemia and only a transient leucopenia and thrombocytopenia appeared in mice exposed to 1,025 r with spleen shielding.[2, 3] This result was attributed to the fact that ectopic blood formation was intensified in the lead-shielded spleen within forty-eight hours after exposure, and recovery of the bone marrow, lymph nodes, and thymus occurred by eight days. In the animals without lead shielding of the spleen, no recovery of hematopoietic tissue or "spotty" foci of recovery only was apparent before death of the animals. No studies have been done yet to determine whether or not a hastening of hematopoietic recovery also occurs in mice exposed to doses of 1,300 r, 1,500 r, or more. This information would be important to the radiation problem since it would indicate whether hematopoietic recovery is independent of survival after exposure to these dosages. Survival roughly parallels hematopoietic recovery with dosages up to 1,025 roentgens. It will be of significance to establish whether hematopoietic destruction is irreversible or still reversible at dosages of 1,300 r, 1,500 r, 2,000 r, or more, since in the case of accidental exposure to penetrating radiations or radiologic warfare the dose sustained may or may not be uniform over the entire body and the ability of surviving hematopoietic tissue to enhance recovery of destroyed tissue or of artificial stimulation (i.e., injection of theoretically potent extracts of hematopoietic tissue) to enhance or produce recovery of hematopoietic tissue will or may depend thereon.

The factor* responsible for the rapid recovery of hematopoietic tissue and reduced mortality in the spleen-shielded mice appears to be emanating from

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*It is obvious that several factors may be involved, but for convenience of expression the singular will be used throughout this dissertation.

TABLE I. SURVIVAL OF MICE EXPOSED TO VARIOUS DOSAGES OF X-RADIATION WITH AND WITHOUT SHIELDING OF SURGICALLY EXTERIORIZED SPLEEN*

WITH LEAD SHIELDING OF SPLEEN	DOSAGE IN R	NUMBER OF MICE USED	NUMBER OF 28-DAY SURVIVORS	SURVIVAL IN %
Yes	1,025	135	105	77.7
No	1,025	273	3	1.1
Yes	1,100	42	23	54.7
No	1,100	12	0	0
Yes	1,200	35	5	14.3
No	1,200	6	0	0
Yes	1,300	74	2	2.7
Yes	1,300†	26	7	26.9
No	1,300	22	0	0
Yes	1,600	46	0	0
No	1,600	—	—	—

*Includes only data of past seven months. Survivors include only animals alive through twenty-eight days after irradiation.

†Technique differed in that small artery at distal tip of spleen was not severed and thus no infarct occurred in spleen.

the shielded spleen. From the experiments already briefly described, the factor might be either cellular or humoral since the circulation of the shielded spleen remains intact and since the shielded spleen so quickly becomes the site of intense ectopic blood formation involving all normal cell types. This phenomenon of hastened hematopoietic recovery in spleen-shielded animals is not specific to the mouse. Lead shielding of the spleen of rats, guinea pigs, and rabbits during exposure to dosages of x-radiation at or above the LD_{50} was followed by a more rapid recovery of hematopoietic tissue than in animals exposed without spleen shielding.[3, 4] It was found, further, that the phenomenon was not specifically dependent on spleen shielding since lead shielding of the appendix of rabbits was also followed by a hastening of hematopoietic recovery.[3, 5, 6] Ectopic blood formation occurs rapidly and intensively in the lead-shielded spleen of mice, rats, and guinea pigs, but only a modest increase in ectopic blood formation occurs in the lead-shielded spleen of rabbits. The lead-shielded appendices of rabbits exposed to dosages of 800 r or 1,000 r whole body x-radiation develop no ectopic blood formation yet recovery of the destroyed hematopoietic tissue occurs more rapidly than in animals without appendix shielding.[3, 5, 6] Thus, the development of major sites of ectopic blood formation is not essential and probably is not the deciding factor in the recovery of the hematopoietic tissue or indeed the survival of the spleen- or appendix-shielded animals. The spleens or the appendices which when shielded produce the factor have lymphocytes and a rich reticular network in common. A number of experiments have been performed to eliminate the migration of a single or several cell types (seeding or colonization) from the lead-shielded tissue as a factor in enhancing recovery of the hematopoietic tissue destroyed by irradiation. These and other experiments are described briefly below. The techniques have been described in detail previously.[1, 2, 3, 5] Since a rather large number of related pilot or preliminary experiments are being presented in this communication, the technique peculiar to the individual experiment as well as the results and discussion will be presented with each experiment in order to maintain continuity.

GENERAL METHODS

The mice used in this study were all CF-1* females and were 10 to 12 weeks of age when the experiments were initiated. The mice were kept in the animal farm in a constant-temperature room (74° F.) for four to six weeks before use and were maintained on a diet consisting of Derwood† and water ad libitum before and after the experimental procedures described below.

Dosimetry.—The x-rays administered in these experiments were generated in a 250 kv. machine operating at 15 milliamperes. A 0.25 mm. copper filter was used. The half-value layer in copper of the filtered beam using 240 kv. was 1.0 millimeter. The exposures were measured with a Victoreen condenser meter equipped with a 250 r chamber. Measurements were made in air at the position occupied by the center of the animal's body. The dose rate averaged 70 r per minute at 55 centimeters.

Groups of mice were exposed to single dosages of 1,025 r, 1,100 r, 1,200 r, 1,300 r, and 1,600 r total body x-radiation with or without lead shielding of the surgically exteriorized liver, intestine, or kidney. Still other groups were exposed to 1,025 r and the head, entire hind limb, or part of the hind limb was lead shielded during exposure. All mice irradiated or operated or both were anesthetized with Nembutal (0.072 mg. per gram mouse) given intraperitoneally. In animals involved in surgical exteriorization procedures, the abdomen was shaved, an incision was made in the appropriate quadrant of the abdomen, and the visceral tissue to be lead shielded or sham shielded was brought out through the incision. In the spleen-shielding experiments the main pedicle of the spleen was left intact, but to facilitate the exteriorization a small vessel at the distal end of the spleen was cut but not tied. Severing this vessel regularly produced an infarct in the tip of the spleen proximal to the vessel. The infarct varied in size usually involving up to one-fourth of the distal tip. In an occasional animal the entire spleen was found to be infarcted in animals sacrificed for histologic study.

During the period in which the exteriorized tissues were outside the peritoneal cavity, they were kept moist with physiologic saline. After the irradiation was completed the visceral tissue was returned to the abdominal cavity and the incision was sutured with silk. Complete recovery from the anesthetic required from two to three hours. The mice were returned to the animal farm and deaths were recorded in twenty-four hour periods through twenty-eight days.

Study of hematopoietic recovery in the experiments described below was carried out separately from the survival studies and consisted of groups of animals sacrificed at intervals after irradiation or allowed to survive for hematologic determinations on the peripheral blood.

RESULTS AND DISCUSSION

Spleen Implant Experiments.—As discussed in the introduction, the spleen and the appendix are rich in so-called reticuloendothelial‡ tissue. An abundance of free hematopoietic cells is available in the shielded spleen or appendix for transport through the blood stream to the destroyed hematopoietic tissue of the irradiated animal. Experiments were designed to minimize the likelihood that such transport or colonization could yield foci from which reconstitution of destroyed hematopoietic tissue might proceed.

Mice were exposed to 1,025 r whole body x-radiation without spleen shielding. At the completion of radiation four spleens (approximate total weight 10 to 20 mg.) freshly removed from 1- to 7-day-old mice were placed

*CF-1 raised by Carworth Farms. Homozygous for the recessive genes aa bb cc.

†Manufactured by the Derwood Mill, Derwood, Md. Consists of skim milk, ground wheat, yeast, corn oil, salt, iron citrate, shark liver oil, and Delsterol.

‡This term is here used to designate all parts of the blood-forming tissue including cells which are potential macrophages such as the Kupffer cells.

into the peritoneal cavity of each of the irradiated mice. The implanted spleens were not anchored within the recipient animal's peritoneal cavity. As is shown in Table II, of thirty-two animals with spleen implantation sixteen

TABLE II. SPLEEN IMPLANT EXPERIMENTS

NUMBER OF MICE USED	WHOLE BODY DOSAGE IN R	SPLEEN IMPLANTS AFTER IRRADIATION (WITHIN ½ HOUR)	NUMBER OF 28-DAY SURVIVORS	SURVIVAL IN %	HEMATOPOIETIC RECOVERY AT 8 DAYS
42	1,025	No	1	2.3	0
32	1,025	4 spleens from 1 to 8 day old mice	16	50	Yes
18	1,025	2 spleens from 4 to 5 wk. old mice	8	45	Not studied
24	1,025	4 spleens from 1 to 8 day old mice implanted 2 days after irradiation	4	16.6	Not studied

or 50 per cent survived the twenty-eight day period of observation. In addition, an experiment was performed in which after exposure of 10- to 12-week-old mice to 1,025 r whole body x-radiation two fresh spleens (approximate weight 0.150 grams) from 4- to 5-week-old mice were dropped into the peritoneal cavity immediately after completion of irradiation. The survival of these mice was materially enhanced. Of eighteen mice thus irradiated and given two spleens, eight survived (45 per cent) (Table II). All that can be expected from the spleen implants during the first few days under these circumstances is a leaking out of "tissue juice"—the free cells in the splenic implants soon become necrotic and histologic reconstitution of splenic tissue does not occur for six days or more if indeed a "take" occurs at all. Takes or recognizable splenic implants were observed in mice beginning on the sixth or seventh day. In approximately 75 per cent of the animals surviving at thirty days, recognizable takes or splenic implants were seen. In the 25 per cent of survivors in which a splenic take was not demonstrated, the possibility exists that the juice from the spleen implants was sufficient to initiate recovery or that temporary "takes" occurred. This interesting observation is being studied more thoroughly. In a few animals which failed to survive, gross evidence of "takes" were not apparent. It seems unlikely that enhancement of survival under these circumstances is due solely to leaking out of juice already present in the donor spleen at the time of removal from the donor baby mouse. A "take" or successful implant necessarily indicates that some cells survive in the implant; that these cells migrate out and are disseminated to the marrow, lymph nodes, and thymus is possible but unlikely. The enhancement of survival may be related to the leaking out of a factor present in the donor spleens when removed from the donor, or production of the factor by surviving cells, or a combination of both. Histologic study of the hematopoietic tissues of mice exposed to 1,025 r without spleen shielding but with baby spleen implants indicates that not only is the mortality reduced but

hematopoietic recovery is enhanced. Beginning recovery of the marrow and lymphatic tissue (lymph nodes) was evident as early as four days in some of the animals sacrificed at this interval. In animals sacrificed at six days the evidence of beginning recovery in the hematopoietic tissue was present in most animals, and in animals sacrificed at eight days recovery was nearly complete or well under way. These facts indicate again that these factors which influence survival and hematopoietic recovery appear to be related at least at this dosage level.

A number of experiments have been performed in which immediately after exposure of 10- to 12-week-old mice to 1,025 r whole body x-radiation, one spleen (weight circa 0.1 gram) freshly removed from a nonirradiated adult mouse was dropped into the peritoneal cavities of the irradiated mice as described. Of twenty-four animals thus treated, one or 4.2 per cent survived (Table III).

TABLE III. SURVIVAL OF MICE EXPOSED TO 1,025 R WHOLE BODY X-RADIATION WITH SPLEEN IMPLANTS FROM ADULT MICE AFTER IRRADIATION

NUMBER OF MICE USED	WHOLE BODY DOSAGE IN R	SPLEEN IMPLANTS WITHIN 10 MIN. AFTER X-RAY	SPLENECTOMY	NUMBER OF 28-DAY SURVIVORS	SURVIVAL IN %
24	1,025	1 adult	No	1	4.2
30	1,025	1 adult*	No	0	0
28	1,025	1 adult spleen	Yes, immediately after irradiation	0	0
47	1,025	Original spleen returned immediately after irradiation†	Yes, immediately before irradiation	4	8.5

*Spleen used for implant was taken from animals two days after exposure to 1,025 r x-radiation with lead shielding of the surgically exteriorized spleen.

†Spleen which was removed immediately before whole body exposure of mouse was kept moist with warm saline during x-ray period and then returned to the peritoneal cavity.

In another experiment spleens from adult mice (10 to 12 weeks of age) which had lead shielding of the spleen during whole body exposure to 1,025 r were removed two days after the spleen-shielding procedure. Immediately after removal a single spleen was dropped into the peritoneal cavity of each adult mouse which had just completed exposure to 1,025 r without spleen shielding. Of thirty animals thus treated, none survived (Table III).

Another experiment was performed which was identical to the one just described except that at the time the donor spleen was dropped into the peritoneal cavity of the recipient, irradiated, non-spleen-shielded mouse, the spleen of the latter was surgically removed. Of twenty-eight animals thus treated, none survived.

Forty-seven adult mice were splenectomized and the spleen was kept moist with physiologic saline during exposure of the splenectomized mice to 1,025 r whole body x-radiation. Immediately after completion of exposure the same spleen was dropped into the peritoneal cavity of the mouse from which it was originally removed. Four or 8.5 per cent of these mice survived

the twenty-eight day period of observation (Table III). Except for the latter experiment, which is questionably significant, adult spleen implants all have been ineffective in enhancing survival. Several factors may be involved and will only be mentioned. Perhaps the fact that this strain of mice is heterozygous influences the reaction of the recipient adult mouse to the transplanted adult spleen. If this were the only factor, however, one would expect that return of the spleen from the same mouse would be more effective, unless leaving the spleen out of the circulation for from twelve to fifteen minutes either destroyed or reduced the factor presumed to be in the spleen. We have not yet sacrificed animals in the adult spleen transplant experiment for gross or microscopic study and therefore do not know whether these implanted adult spleens actually vascularize and again become functional spleens. Another possibility is that when adult spleens were used they were implanted intact without cutting the surface and this may have interfered either with early vascularization or escape of tissue juice or both. In contrast to the adult spleen experiments, when baby spleens (from 1-day-old to 5-week-old mice) were implanted, they were invariably partially torn or cut in the process of transplantation. We are currently doing spleen transplant experiments with highly inbred mice and are, in addition, deliberately making multiple incisions in the transplanted spleens or actually cutting the spleens in several pieces at the time of transplantation to facilitate escape of tissue juices and vascularization.*

It is possible perhaps that the adult spleen is not as rich in the factor and must remain directly in the circulation during the early postirradiation period to provide a sufficient quantity of this factor to initiate the recovery process. Some observations made in the process of the various experiments may be of interest in this connection. As was mentioned in the section on General Methods, complete infarction of the spleen occasionally occurs in the shielding technique. It was found that in the mice exposed to 1,025 r with spleen shielding which were sacrificed for histologic study, a hastening of hematopoietic recovery was evident in the bone marrow and mesenteric lymph nodes by eight days even though the spleen was obviously completely infarcted.

Another observation which has bearing on this problem is the fact that, if the vessel at the distal tip of the spleen is not severed and thus no infarct occurs, the survival of mice exposed to 1,300 r whole body x-radiation is definitely higher than in mice in which this vessel is severed (Table I). Two spleens from mice 7 to 12 days old implanted into irradiated adult mice are not effective in enhancing survival and hastening hematopoietic recovery, whereas four spleens from mice 7 to 12 days of age are effective in enhancing survival of adult mice exposed to 1,025 r (Table IV).

Liver-Shielding Experiments.—Additional experiments have been done which indicate more conclusively that the factor involved is humoral rather

*Since submission of this manuscript it has been found that incising the capsule of the adult spleen before implantation is effective. Circa 50 per cent of mice survive 1,025 r whole body x-radiation if a single adult spleen with an incised capsule is implanted immediately after irradiation.

TABLE IV. SURVIVAL OF MICE EXPOSED TO 1,025 R WHOLE BODY X-RADIATION WITH TWO AND FOUR SPLEEN IMPLANTS AFTER IRRADIATION

NUMBER OF MICE USED	WHOLE BODY DOSAGE IN R	SPLEEN IMPLANTS WITHIN 10 MIN. AFTER IRRADIATION	AGE OF SPLEENS IMPLANTED	NUMBER OF SPLEENS IMPLANTED	NUMBER OF SURVIVORS (30 DAYS)	30-DAY SURVIVORS IN %	HEMATOPOIETIC RECOVERY IN 8 DAYS
14	1,025	None	–	–	0	0	None
8	1,025	Yes	7-12 days	4	2	25	Not studied
12	1,025	Yes	7-12 days	2	0	0	Not studied
23	1,025	Yes	3-6 days	4	4	17.4	Not studied

than related to cellular "seeding" or "colonization." Using a technique similar to that of the spleen-shielding experiments, the livers of mice were exteriorized and a volume equal to about eight times that of the mouse spleen was lead shielded during exposure of the animals to 1,025 r whole body x-radiation. Observations were made on the effect of this procedure on hematopoietic recovery. Histologic studies on the hematopoietic system conclusively showed that hematopoietic recovery was hastened, as compared with animals exposed to this dose without liver shielding. However, the enhancement or hastening of hematopoietic recovery did not seem to attain the same degree as that observed after lead shielding of the spleen. These observations supplement the baby spleen implant data in that further evidence is added to indicate that the phenomenon or factor responsible for enhanced survival and hastened hematopoietic recovery is probably humoral. We recognize the possibility that lymphocytes may migrate from the portal areas in the liver and, therefore, the liver-shielding experiment does not entirely exclude cellular "seeding" or "colonization" as a factor in hematopoietic regeneration. One of the bits of evidence which argues strongly against cellular "seeding" from the shielded tissue as the basis for recovery of the irradiated hematopoietic system is the observation already reported[3] that regeneration in the bone marrow from osteoblasts and reticular cell transformation is striking. Thus cellular seeding and proliferation of the colonizing cells at best could be considered only a part of the repair process. Although no experiments specifically designed to obtain data on the effect of liver shielding on survival have as yet been done, it was evident from the number of animals surviving at late intervals for autopsy and histologic data that survival was enhanced. In another experiment in which the liver was lead shielded as before and the animals were observed for hematologic changes, five of fifteen animals (33 per cent) survived through the twenty-eighth day (Table V).

Intestine, Kidney, Head, and Leg Shielding Experiments.—Lead shielding of the surgically mobilized intestine of mice exposed to 1,025 r likewise enhances survival and hastens hematopoietic recovery (Table V). The intestinal tract is rich in lymphatic tissue. Thus all the tissues which produce this phenomenon when lead shielded have the one common feature—that of reticuloendothelial constituents. If, in further experiments, the lead-shielded spleen continued to be more effective than lead shielding of roughly equal volumes of other tissues such as the liver, intestine, and the like, it could perhaps easily

TABLE V. SURVIVAL OF MICE EXPOSED TO 1,025 R X-RADIATION WITH LEAD SHIELDING OF VARIOUS TISSUES

NUMBER OF ANIMALS	WHOLE BODY DOSE IN R	TISSUE LEAD SHIELDED	NUMBER OF SURVIVORS	SURVIVAL IN %	HEMATOPOIETIC RECOVERY IN 8 DAYS
135	1,025	Exteriorized spleen (0.1 Gm.)	105	77.7	Complete (++++)
93	1,025	None	0	0	0
15	1,025	Exteriorized lobe of liver (0.8 Gm.)	5	33	Nearly complete (+++)
15	1,025	None	0	0	0
15	1,025	Exteriorized intestine (2.5 Gm.)	4	26.6	Nearly complete (+++)
15	1,025	None	0	0	0
18	1,025	Head (3.0 Gm.)	5	27.7	Only partial (+)
12	1,025	None	0	0	0
15	1,025	Right hind limb (including thigh) 1.5 Gm.	2	13	Not studied
15	1,025	Right hind foot (0.1 Gm.)	1	6.6	Not studied
28	1,025	Exteriorized right kidney (0.19 Gm.)	0	0	0
8	1,025	None	0	0	0

be explained on the basis of the fact that the spleen contains a much higher proportion of reticuloendothelial tissue than either the liver or intestine. Lead shielding of a single kidney in mice fails to materially enhance survival or hastens hematopoietic recovery. The weight of one kidney is slightly more than the weight of the spleen. Lead shielding of the head of the mouse during exposure to 1,025 r whole body x-radiation does definitely enhance survival. Of eighteen animals thus exposed, five or 27.7 per cent survived. Lead shielding of the entire hind leg of the mouse during exposure of the balance of the body to 1,025 r enhances survival slightly. Lead shielding of the head or of the leg, however, provides shielding for skin and muscle as well as hematopoietic tissue, and thus this latter type of shielding experiment is too nonspecific for adequate interpretation. It is possible that lead shielding of any tissue in sufficient quantity might produce this phenomenon—practically all tissue in the body contains mesenchymal tissue.

Studies on "Prophylactic" versus "Therapeutic" Attack on Radiation Injury.—Several authors have reported studies indicating that the mortality from radiation can be materially reduced[7-10] by a variety of means. Glutathione or cysteine administered immediately before irradiation will reduce the mortality; placing the animal in a reduced O_2 tension during exposure to radiation will markedly reduce mortality; cyanide intoxication during exposure to radiation will reduce mortality. These substances or techniques have one very significant aspect in common: they are "prophylactic measures." Administration of glutathione, cysteine, cyanide, or reduced O_2 tension after irradiation has no beneficial effect on survival. With these facts in mind attempts were made to determine whether the phenomenon occurring after spleen shielding occurred as a result of presence of the "factor" during irradiation or following irradiation. Two approaches were made. In one, the

intact spleens from baby mice were placed in the abdomen of mice irradiated with 1,025 r whole body x-radiation as described. This technique enhanced survival and hastened hematopoietic recovery as well.

In another experiment the pedicles of the shielded spleens were tied off with silk during the period of irradiation and immediately released after completion of irradiation. Of fourteen mice irradiated with 1,025 r whole body x-radiation with spleens lead shielded as well as having the circulation to the spleen occluded during irradiation, nine or 64 per cent survived. Not only was survival enhanced but hematopoietic recovery was hastened as in animals with spleen shielding only. Thus the presence of the factor is not necessary during irradiation and may, therefore, be considered as being of significance in a therapeutic sense.

Splenectomy Experiments.—In an attempt to get some concept of the period during which the lead-shielded spleen must remain intact in the post-irradiation period to exert a maximum effect on the enhancement of survival and on the hastening of hematopoietic recovery, the following experiments were done. Mice were exposed to 1,025 r whole body x-radiation with lead shielding of the spleen. At various intervals after irradiation, as is shown in Table VI, the spleens were removed and studies were made on survival and

TABLE VI. SURVIVAL OF MICE EXPOSED TO 1,025 R WITH LEAD SHIELDING OF THE SURGICALLY EXTERIORIZED SPLEEN, WITH REMOVAL OF THE SPLEEN AT VARYING INTERVALS AFTER X-RAY

TIME OF SPLENECTOMY AFTER X-RAY	NUMBER OF MICE	SURVIVAL IN %	DOSAGE IN R
1 hr.	41	73.4	1,025
6 hr.	13	46.2	1,025
12 hr.	66	39	1,025
1 day	27	85	1,025
2 days	40	55	1,025

hematopoietic recovery. It is evident that leaving the lead-shielded spleen in the circulation for as short a time as one hour has a definite beneficial effect on survival and hematopoietic recovery is also hastened. These data would appear to indicate that leaving the spleen in the circulation for one to six hours is as effective as forty-eight hours. It would appear from these data that the therapeutic effect derived from the shielded spleen has already been exerted in a few hours and its removed thereafter does not interfere materially with the recovery process. Perhaps the recovering hematopoietic tissue can by this time carry on the manufacture of the factor necessary for rapid and orderly hematopoietic recovery.

Delayed Implantation of the Spleen.—An important aspect of recovery from irradiation injury which the foregoing experiment does not answer is: would the addition of the "factor" for the first time at one, two, three, or more days after irradiation still improve survival and enhance hematopoietic recovery? In other words, would the late addition of the factor be effective or must it be present soon after irradiation injury? This problem can be answered by (1) implantation of spleens into irradiated animals at intervals after irradia-

tion, and (2) experiments with injection of spleen extracts, embryonic juice, and the like. These studies are in progress. In preliminary studies mice were irradiated with 1,025 r whole body x-radiation, and two days after irradiation, four baby spleens were implanted into the peritoneal cavity. Of twenty-four mice thus treated, five or 20 per cent survived twenty-three days and four or 16.6 per cent survived beyond twenty-eight days (Table II). These data indicate that "late" addition of the factor is at least partially effective in enhancing survival.

Radiation Suppression of Spleen Factor.—Experiments were done to determine the amount of radiation necessary to suppress the production of the factor responsible for the phenomenon under discussion. Using the technique discussed, mice were exposed to 1,025 r whole body x-radiation with lead shielding of the spleens. During irradiation the shield was removed from the spleens and thus a predetermined increment of the radiation dose to the body was delivered to the spleen. In this way dosages of from 50 to 600 r inclusive were delivered to the spleen, whereas the balance of the mouse sustained a dose of 1,025 r. Survival data on a limited number of animals indicate that even with 600 r to the spleen survival is enhanced (Table VII).

TABLE VII. PARTIAL SPLEEN-SHIELDING EXPERIMENTS

NUMBER OF ANIMALS	WHOLE BODY DOSAGE IN R	INCREMENT TO SPLEEN IN R	PER CENT SURVIVING AT 28 DAYS	HEMATOPOIETIC RECOVERY AT 8 DAYS
24	1,025	0	0	Complete (++++)
24	1,025	50	–	Nearly complete (+++)
24	1,025	100	–	Nearly complete (+++)
29	1,025	200	48.2	Partial (++)
24	1,025	300	50.0	Partial (++)
16	1,025	400	–	Study
15	1,025	500	–	not
14	1,025	600	21	complete

Histologic studies completed thus far show that hastening of hematopoietic recovery still occurs if dosages up to and including 200 r are given to the spleen and a dose of 1,025 r is sustained by the balance of the body. These findings are of considerable interest since they imply that if sufficient reticuloendothelial tissue in the body is not irradiated or receives 200 r or less, the speed of recovery of the destroyed tissue may be enhanced by the surviving tissue. Since it seems to be clear that free hematopoietic cells such as the lymphocytes are not necessary for the production of the factor and if one assumes that fixed reticuloendothelial cells are responsible for the production, it may be suggested that functionally these latter cells are not materially altered with dosages of 200 r or less. Whether the factor responsible for the hastening of hematopoietic recovery is suppressed with doses above 200 r is now under investigation.

Review of Histologic Changes in Shielded Spleen.—The histologic findings in the lead-shielded spleens of mice exposed to 1,025 r whole body x-radiation should perhaps be given special mention. Originally it was observed in mice given Sr^{89} (2 microcurie per gram) that whereas the bone marrow was de-

stroyed, the spleen showed intense ectopic blood formation at three days.[111] In these spleens the white pulp had largely disappeared by three days after Sr^{89} injection and the red pulp in many instances had largely replaced the white pulp and extended up to the central arteriole of the original white pulp. This ectopic blood formation was largely erythroblastic under these circumstances at the three-day post-Sr^{89} injection interval. In the lead-shielded spleen of irradiated animals the cellularity of the white pulp is markedly reduced by twenty-four hours. Often no lymphatic nodules remain, the lymphocytes are markedly reduced or virtually absent, and the white pulp as a whole consists of a small nest of reticular cells about the central arteriole. In some specimens it is difficult to find lymphatic tissue in the entire spleen at the forty-eighth hour interval. By twenty-four hours erythroblasts have increased within the red pulp and islands of erythroblasts may be seen within the white pulp. Occasionally at the forty-eight hour interval the spleen appears to consist almost exclusively of erythroblasts. Beginning at about four to five days, megakaryocytes appear in the expanded red pulp and increase rapidly in number thereafter. Granulocytopoiesis begins to appear about the sixth day and increases in amount thereafter. Beginning about the eighth day the lymphatic tissue in the splenic white pulp begins to reaccumulate. This reaccumulation of lymphocytes seems to correlate with the recovery of the bone marrow and a reduction in the number of erythroblasts in the splenic pulp. That this disappearance or reduction in splenic white pulp is probably not a specific "indirect effect" of radiation is shown by the fact that in rabbits exposed to whole body x-radiation with spleen shielding, the white pulp of the spleen is not correspondingly reduced. In the rabbit with appendix shielding the lymphatic tissue in the shielded appendix does not appear to be reduced.[6] Ectopic blood formation is minimal in the lead-shielded spleen of rabbits and absent in the lead-shielded appendix of rabbits and thus competition between erythroblast proliferation and lymphocyte proliferation is not a factor. Returning to the shielded mouse spleen, it is interesting to speculate about the disappearance of lymphatic tissue, the inhibition of its return, and the relative tardiness of the appearance of ectopic megakaryocytopoiesis and granulocytopoiesis, whereas erythroblasts "take over" the organ. This differential suppression of cell types cannot be explained adequately at present. One might have anticipated an early increase in ectopic granulocytopoiesis rather than erythrocytopoiesis on the basis of need. No significant reduction of circulating erythrocytes had occurred in two or three days, whereas leucopenia is already severe by this interval. Thus no apparent stimulus is present for the major intensification of erythropoiesis. Perhaps under these circumstances the organ reverts largely to an embryonic state which, until late in embryonic life, consists exclusively of erythroblasts.

The Spleen and Nitrogen Mustard Toxicity.—In order to determine whether the beneficial effect of spleen shielding was specific only for radiation injury, a preliminary experiment was designed to test the effect of a comparable technique on nitrogen mustard destruction.[12] Mice of the same age and strain employed in the irradiation experiments described were anesthetized

with Nembutal (dose 0.072 mg. per gram) and while under anesthesia the spleen was exteriorized surgically. The splenic pedicle was then tied with silk and nitrogen mustard [methyl-*bis* (β chloroethyl) amine hydrochloride] in a dose of 0.003 to 0.005 and 0.010 mg. per gram was given intravenously via the tail vein. After ten to fifteen minutes the tie on the splenic pedicle was released and the abdominal incision closed. Controls were given Nembutal anesthesia and nitrogen mustard. No attempt was made to study the influence of this technique on survival. Recovery of the lymphatic tissue and bone marrow of the mice in which the splenic pedicle was tied off during and ten to fifteen minutes after HN_2 administration preceded the recovery of these tissues in animals with an intact splenic circulation during and after HN_2 administration. (It is well known that the major action of HN_2 occurs within ten to twenty minutes after administration.[13, 14]) The spleen of the animals without a pedicle tie underwent the typical destruction and atrophy seen in irradiated mice. The spleen of mice with the splenic pedicle tied like the lead-shielded spleens of irradiated mice increased in size and became the site of major ectopic blood formation. This finding is of extreme interest because it indicates that the phenomenon is not specific only for the hastening of hematopoietic recovery from the injury produced by irradiation but appears to be effective as well in hastening recovery from the destruction induced by HN_2 administration. Karnofsky and associates[13] reported that occluding the arterial blood supply to the lower extremities of the rat for fifteen minutes during and after nitrogen mustard administration reduced the severity of the hematologic depression characteristically observed in intact animals given the drug. Histologic studies were reported to show that the destruction and atrophy of the hematopoietic tissue proximal to the occluded arteries occurred, whereas a much decreased destructive effect was apparent in the bone marrow of the lower extremities whose arterial supply had been occluded during the HN_2 administration. The fact that a lesser hematologic depression occurred in the artery-occluded animals was attributed to the fact that marrow of the extremities remained essentially normal or actually hypertrophied. Unfortunately, histologic observations beyond four days were not studied or at least were not reported in these two experimental groups. If recovery of the destroyed blood-forming tissue occurred earlier in the aorta-occluded group, it probably would have been apparent after the four-day interval.

Effect of Spleen Shielding on Recovery of Tissues Other Than the Hematopoietic System.—The effect of spleen shielding on the recovery of tissues other than the hematopoietic system is, in some respects, difficult to interpret adequately. The recovery of the intestinal tract is definitely hastened in mice, rats, and rabbits which have had spleen shielding during irradiation. The gross pathologic findings of ulceration, petechiae, gross hemorrhage, blood tinged mucus, and evidence of diarrhea which are described in the post-irradiation period in animals exposed to dosages of whole body x-radiation in the LD_{50} range or above are less frequently encountered in the spleen-shielded animals. Histologic recovery of the lymphatic tissue in the intestinal wall of the small

and large intestine occurs with the same rapidity as that of the peripheral lymph nodes in spleen- or liver-shielded animals, and there is little early or late evidence of denuding of epithelium, hemorrhage, or ulceration. Whether this rapid recovery of the intestinal tract and actual reduction in the expected pathologic changes in the gastrointestinal tract is a result of some factor reducing the severity of damage or rather some factor which hastens recovery remains to be proved. It seems not at all unlikely that the rapid recovery of hematopoietic tissue and the presence of a "normal," functioning spleen may well influence the ulceration and bacterial invasion and thus reduce "radiation sickness," by septicemia, and death. Liver shielding and head shielding likewise favorably influence recovery of the intestinal tract.

Preliminary histologic studies on the ovaries and testes of irradiated mice and rabbits with and without spleen shielding have failed to show any material difference in the degree of destruction or speed of recovery.

Comments on Relationship of Hematopoietic Recovery and Enhanced Survival.—It is probably premature at this time to assume that following spleen shielding a single factor is responsible for enhanced survival of animals, for the rapid recovery of the hematopoietic system, and for the enhanced recovery of gastrointestinal tissue. On the other hand, it seems logical that recovery of the hematopoietic system may be instrumental in averting fatal septicemia, may promote healing, or even prevent ulceration of the gastrointestinal tract, and reduce or eliminate hemorrhage. Hematopoietic recovery from radiation injury is hastened in guinea pigs, rats, and rabbits by spleen shielding,[1, 3, 4] but survival is not enhanced by a factor of two as is true in mice. One can only speculate at the moment as to whether the enhanced survival from whole body irradiation, which follows liver shielding, intestine shielding, or head shielding, is the result of the same mechanism as spleen shielding.

Whether the factor (or factors) involved is due to cell migration from the shielded tissue or dispersion of a noncellular substance is not yet proved. The evidence presented in this paper points in the direction of a humoral noncellular substance which stimulates regeneration of the hematopoietic tissue or supplies something which makes repair and regrowth possible. The possibility that irradiation produces a "toxin" which inhibits regeneration of irradiated tissue and that the shielded tissue neutralizes such a toxin has not been entirely disproved by the experiments reported herein.

The possibility that irradiated tissue can be stimulated to an earlier recovery than previously supposed by post-irradiation "therapy" is encouraging. The potential significance of these findings to the problem of radiation injury and to certain other clinical syndromes is thought provoking.

SUMMARY AND CONCLUSIONS

The LD_{50} per twenty-eight days for spleen-shielded mice is circa 1,100 r whole blood x-radiation compared to circa 550 r for mice without spleen shielding. Intact mice rarely survive 800 r whole body x-radiation. Only 1.1 per cent survive 1,025 r whole body exposure without spleen shielding.

Recovery of hematopoietic tissue as well as the recovery of the gastrointestinal tract is significantly hastened by spleen shielding. Lead shielding of part of the exteriorized liver, the exteriorized intestine, or the entire head or the hind limb enhances survival of animals exposed to 1,025 r whole body x-radiation but not to the extent observed with spleen shielding. Shielding of a single kidney during exposure to this dosage does not enhance survival.

The survival of mice in which the circulation of the shielded spleen is clamped off during exposure of the animal to 1,025 r and the clamp is released immediately thereafter is approximately the same as the survival in the regular procedure of spleen shielding only.

Surgical extirpation of the shielded spleen at intervals after the shielding procedure indicates that a beneficial effect has been exerted even if the spleen has been left intact in the circulation for one hour. The survival of animals exposed to 1,025 r with spleen shielding and subjected to splenectomy from one to forty-eight hours later is between 39 and 85 per cent for the twenty-eight day period of observation.

Transplantation of one to four fresh spleens from young mice into the peritoneal cavity of mice immediately after exposure to 1,025 r whole body x-radiation significantly increases the survival of the irradiated mice. Similar transplantation of spleens into irradiated mice two days after whole body exposure to 1,025 r x-radiation likewise enhances survival but is definitely not as effective.

These facts indicate that unlike glutathione, cysteine, O_2 deprivation, or cyanide intoxication, the "factor" in spleen shielding which effects a significant increase in survival of mice from whole body x-radiation is unnecessary during the actual irradiation process and is definitely effective after irradiation.

In the experiments described, the general assumption is made either that (1) the shielded tissues produce a substance of a noncellular nature which exerts a beneficial effect on the recovery of the irradiated animal or (2) that cells migrate from the shielded tissue which enhance recovery of the irradiated tissues. The evidence appears to be in favor of the first assumption but both may be wrong. The possibility has not been eliminated that irradiation of tissue produces a "toxin" and that the shielded tissues or implanted tissues in some way exert a detoxifying action as the "toxin" courses through the shielded tissues.

The potential significance of these observations to the therapy of radiation injury and other clinical syndromes is discussed briefly.

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THE RESPONSE OF SERUM GAMMA GLOBULIN LEVEL AND COMPLEMENT TITER TO ADRENOCORTICOTROPIC HORMONE (ACTH) THERAPY IN LUPUS ERYTHEMATOSUS DISSEMINATUS

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BOSTON, MASS.

SINCE much interest has been expressed in the possible role of allergic phenomena in the etiology of lupus erythematosus disseminatus, we have felt that it would be of value to report some of our recent experience in following serum complement titers and gamma globulin levels in this disease during its response to adrenocorticotropic hormone (ACTH). A brief description of Case D. B. has been reported previously.[1]

MATERIALS AND METHODS

Clinical Material.—Four patients with lupus erythematosus disseminatus, two of whom were acutely ill (Patients D. B. and G. J.), were studied before, during, and after one or more treatment periods with ACTH. ACTH was given intramuscularly in divided dosage every six hours. Total daily dosage varied from 40 to 200 milligrams.

Methods.—Gamma globulin levels were done by a modification[2] of Kunkel's flocculation technique.[3] Values of 70 units or more are considered definitely elevated. Complement titers were measured by a modification[4] of Heidelberger's method[5] for determining the 50 per cent hemolytic end point. Values below 80 are abnormally low. Anticomplementary titers were done on sera taken from deep freeze storage. One milliliter aliquots of serial twofold dilutions of the heat-inactivated§ specimens were added to 0.5 ml. portions of sensitized sheep cells prepared as for routine complement titrations[4]; 0.5 ml. of 1:300 dilution of active guinea pig serum was added to each tube as complement. The whole was incubated and the amount of hemolysis read as in the complement titration. The amount of guinea pig serum used was ordinarily sufficient to give between 50 and 100 per cent hemolysis of the cells in the system.

RESULTS

In all four of the patients studied, the gamma globulin levels were elevated, as is shown in Table I. However, there was no correlation between the degree of elevation of the gamma globulin and the severity of the disease. On the contrary, Patient G. J., who had a very active process, had the lowest gamma globulin level. It is probably more significant that Patient G. J. was the patient whose disease was of shortest duration. She had been sick for only a matter of a few weeks before the institution of therapy. In all instances, ACTH treatment was marked by a depression of the gamma globulin level to or toward

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§56° C. for thirty minutes.

TABLE I. THE EFFECT OF ACTH TREATMENT ON SERUM COMPLEMENT ACTIVITY AND GAMMA GLOBULIN LEVEL

PATIENT	COMPLEMENT			GAMMA GLOBULIN	
	START	END	AFTER	START	END
D. B. 1	20	30	90	120	90
2	80	60	75	90	70
3	46	30	82	140	88*
G. J.	25	126	196	70	41*
M. A.	85	51	98	197	105
B. V.	76	46	106	110	60

Complement is expressed as 50 per cent hemolytic units per milliliter, gamma globulin as flocculation units per 0.1 milliliter.

*These values represent the low levels to which the gamma globulins fell in these two instances prior to escape from the effect of ACTH.

normal. In Fig. 1 is shown the electrophoretic pattern on Patient D. B. before and after the first two courses of ACTH. The marked reduction in the gamma globulin level is readily evident.

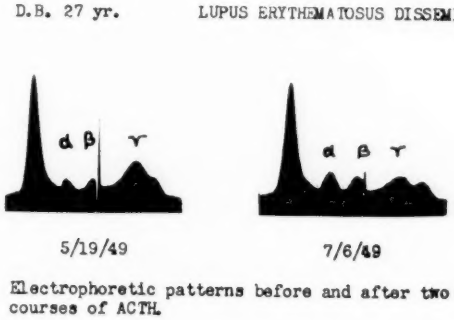

Fig. 1.—Reduction of the gamma globulin by ACTH in lupus erythematosus disseminatus.

In the two who had the greatest degree of activity (Patients D. B. and G. J.)—the activity being judged by the degree of elevation of body temperature and the general appearance of "toxicity"—the complement titer was grossly depressed before therapy. In each instance the end of a treatment period was marked by a sharp upswing in the complement titer. In Patient G. J. there was an additional rise in the midst of a course of therapy which was continuous for over two months. This rise was noted on the thirty-first day and is incorporated in the value of 126 noted at the end of her treatment period. Fig. 2 illustrates the changes in Patient D. B. during several courses of ACTH. A roughly reciprocal relationship between the gamma globulin level and the complement titer is apparent. In addition, the persistent tendency for the rise in complement to occur only at the end of a course of therapy is well illustrated.

In the two relatively inactive patients the initial complement titer was either low normal (Patient M. A.) or only slightly depressed (Patient B. V.). During the course of hormone therapy in these cases, the complement activity fell significantly, in a manner similar to that found in rheumatoid arthritis[4] where we have speculated on the possible depressant effect of ACTH on the formation of complement protein. The post-treatment rises in titer in these two patients were not so spectacular as those noted in the more active cases.

Since it has been shown by Davis and collaborators[6] that the gamma globulins of normal human sera are anticomplementary, some estimate of the role of this factor was made by doing anticomplementary titers on serum samples from Patients D. B. and G. J. (Table II). It is evident from this data that there is very little change in the anticomplementary activity of these sera in dilutions of 1:20 or greater, which were the dilutions used in all determinations of complement titer.

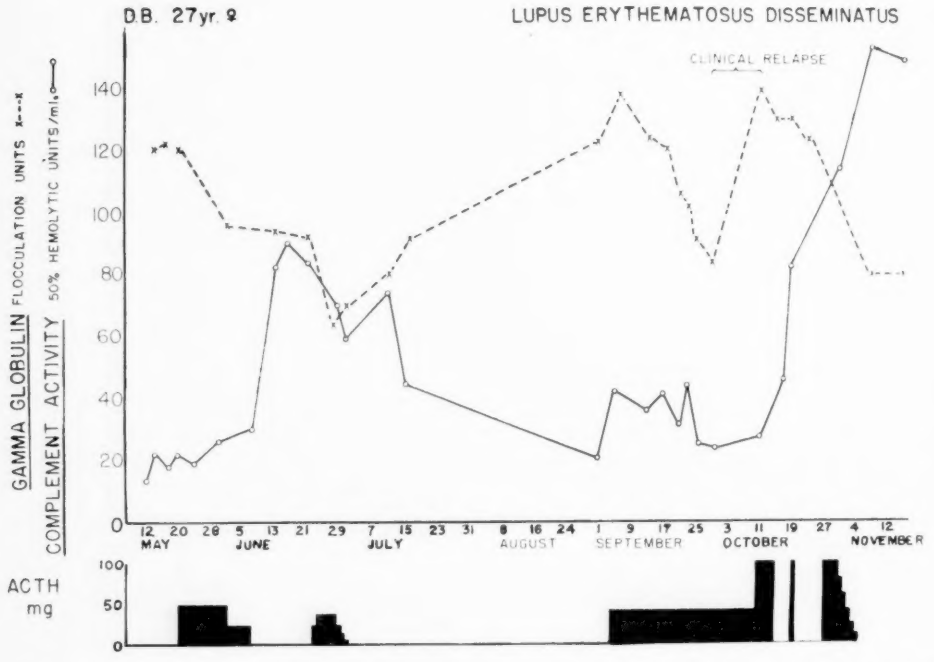

Fig. 2.—The inverse relationship between complement and gamma globulin during ACTH therapy in lupus erythematosus disseminatus. The tendency for the complement titer to rise only at the cessation of therapy is illustrated.

DISCUSSION

The changes in complement titer that we have observed can be interpreted as the resultant of two forces: (1) a tendency for active lupus erythematosus disseminatus to have a lowered serum complement titer and for suppression of this activity to be characterized by a rising titer, and (2) a primary depressant effect of ACTH on complement activity, perhaps by inhibiting its formation. This latter effect would be consistent with the known "antianabolic" activity[7] of the cortical steroids. Thus the complement titers of Patients D. B. and G. J. were very low initially and tended to rise as the disease activity was suppressed by the hormone therapy. In Patient G. J. this rise managed to express itself after prolonged therapy, but in all other instances the presence of the ACTH prohibited this rise until the cessation of treatment. In the two patients with rather quiescent disease, the complement titers were very little depressed by the disease process, and in them we can see the influence of ACTH itself upon

TABLE II. ANTICOMPLEMENTARY TITERS BEFORE, DURING, AND AFTER ACTH THERAPY

PATIENT	DATE	0%*	SERUM DILUTION 1:5	1:10	1:20	1:40	1:80	100%†
D. B.	5/10	10	9	77	87	88	98	135
	6/7	–	55	96	100	112	93	–
	6/16	–	65	96	101	100	104	–
G. J.	7/27	–	35	97	100	103	101	–
	8/26	–	51	87	97	99	93	–
	9/2	–	51	88	95	101	100	–

Numbers refer to readings on the Klett-Summerson colorimeter. Complete suppression of complement activity is represented by the 0 per cent control*, uninhibited complement activity by 100 per cent control.†

complement titer in bolder relief. In Patient M. A. the ACTH effect was essentially the only effect seen, but in Patient B. V., who developed a higher titer after treatment than she had had previously, it would appear that her disease process had contributed a significant element of depression to the pretreatment titer. These interpretations are consistent with the observation that Patient M. A. had the most inactive case, the only evidence of the continued presence of the disease being that of a mild continuing anemia.

It is possible that the apparent reciprocal relationship of complement activity to gamma globulin level reflects immune phenomena of importance in this disease. A close association of the gamma globulins with the immune bodies of the blood has been well established.[8] Furthermore, numerous workers in experimental anaphylaxis[9] and in various diseases known to be of allergic origin[10] have shown that depressions in the level of blood complement do occur during the height of the antigen-antibody activity. Therefore, one could easily conclude that our data indicate similar mechanisms at play in active lupus. That the rises in complement titer do not represent merely alterations in the anticomplementary activity of the serum as a part of the fall in gamma globulins is suggested by our titers of serum anticomplementary activity indicated in Table II. Additional data suggesting that the gamma globulins alone are not responsible for the changes in the complement activity are provided by our observations of these levels during ACTH therapy in rheumatoid arthritis.[2, 4] In this disease we have observed similar or much larger reductions in gamma globulin level with changes in complement titer that do not parallel these in active lupus erythematosus.

SUMMARY AND CONCLUSIONS

In a study of four cases of lupus erythematosus disseminatus, estimations of the serum complement activity and gamma globulin level have been made.

In these cases, disease activity was roughly correlated with degree of depression of complement activity and duration of disease with degree of elevation of gamma globulin content.

During ACTH therapy the complement titer tends to rise and the gamma globulin level to fall.

The possible significance of the observed changes is considered.

The authors are grateful to Dr. Walter Lever of the Department of Physical Chemistry, Harvard Medical School, for the electrophoretic data reported here.

The technical assistance of Miss Elizabeth Geiler and Mr. Jack Kasarjian was greatly appreciated.

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IN VIVO AND IN VITRO EFFECT OF AUREOMYCIN HYDROCHLORIDE ON SYRINGOSPORA (MONILIA, CANDIDA) ALBICANS

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CLINICALLY, aureomycin, the golden crystalline antibiotic, obtained from *Streptomyces aureofaciens*, has been found to be effective in a number of diseases caused by both gram-positive and gram-negative bacteria and of value in some nonbacterial diseases. Like other antibiotics, however, it is not devoid of toxicity which may be acute, subacute, or chronic. In general, the reports in the literature indicate that the common toxic reactions to the drug are nausea, vomiting, headache, epigastric pain, and looseness of bowels or persistent diarrhea. These are common findings in a fair percentage of patients who take the drug orally. Sanders, Rumball, Solomon, Soret, and Ricci[1] reported phlebitis as a sequela of intravenous therapy in 30 to 40 per cent of 116 cases studied. Brainerd, Lennette, Meiklejohn, Bruyn, and Clark[2] reported that when aureomycin was dissolved in Sorenson's phosphate buffer (pH 7.4), combined with 2 per cent procaine, and injected intravenously, there developed invariably a severe, painful, local reaction.

In addition, there has been reported an abrupt rise in temperature occasionally accompanied by a picture simulating shock with a drop in blood pressure and tachycardia.[1, 3] Skin rashes following aureomycin therapy have been described as maculopapular, urticarial, or scarlatiniform.

These toxic manifestations in most instances were not serious enough to warrant discontinuing the aureomycin therapy.

There have been additional side reactions which have a great deal of significance concerning the main thesis of this paper. In a study of 110 patients with chronic brucellosis who were given aureomycin hydrochloride orally, Harris[4] found that side effects occurred in forty-eight of the seventy-nine female patients and in six of the thirty-one male patients. The outstanding symptoms were headache, nausea, vomiting, epigastric pain, persistent diarrhea and anal irritation (with fissuring in two) in both sexes. Bladder irritation occurred in eight, fleeting urticaria in three, dermatitis of the hands in two, and slight generalized desquamation of the epidermis in two. In addition, however, he noted the occurrence, within three days in some patients, of mucous membrane manifestations of the mouth, throat, and pharynx with perlèche-like lesions in twelve women and in one man. Vaginitis occurred in all of the twelve female patients with slight bleeding in two.

Brainerd and associates noted "black tongue" in two of their patients and glossitis in association with skin rashes in two others. Erythema and pruritus of the perineal area occurred in four patients.

Studies, observations, and reports from the Dermatological Department of the Barnard Free Skin and Cancer Hospital and the Department of Medicine, Division of Dermatology, School of Medicine, Washington University. Service of Richard S. Weiss, M.D.
Received for publication, Dec. 20, 1950.

Meiklejohn and Shragg,[5] in treating primary atypical pneumonia with aureomycin, noted in one patient cheilitis and smoothness of the tongue four days after aureomycin therapy was begun.

Black tongue and disseminated crural lesions following aureomycin therapy have been brought to my attention by various dermatologists. Black tongue has also been noted following penicillin therapy.

These various phenomena, the mucous membrane manifestations, perlèche-like lesions, vaginitis, black tongue, glossitis, cheilitis, smooth tongue, perineal and perianal pruritus are often characteristic of moniliasis. In his report, Harris presumed that the high acidity in the gastrointestinal tract resulting from the administration of aureomycin might account for some of the gastrointestinal and bladder manifestations, with a destruction of the normal intestinal flora and/or allergy explaining the mucosal and dermal reactions. He noted also that previously existing symptoms were temporarily aggravated in many patients. He has since found that cultures from these patients showed an increased number of monilial colonies. It may be further presumed that the aureomycin in destroying the normal bacterial flora had allowed the yeastlike organisms to grow more freely and abundantly. On the other hand, does aureomycin exert a stimulatory effect on these organisms? It is this possibility with which this paper is concerned.

My interest in the action of aureomycin hydrochloride on monilial organisms was first aroused in July, 1949, when a woman with a severe cough was referred to me for sputum cultures. In the absence of any other demonstrable pathogens, it was felt that pulmonary mycosis should be considered.

REPORT OF CASE*

Patient H. A., a 40-year-old white woman, a medical technician, complained of a cough, respiratory infection, low-grade fever, and loss of weight. The patient dated her illness from September, 1945, at which time there was an onset of general malaise and loss of weight associated with a low-grade fever. Physical examination by an internist revealed negative findings. There was a question of undulant fever but this was not proved. The symptoms persisted throughout the next two months and in November, 1945, she resigned her position as technician in a private hospital. X-rays made of the chest in November, 1945, revealed nothing unusual.

The symptoms subsided during December, 1945, but reappeared in January, 1946, and persisted until April, 1946, when they again disappeared during bed rest while the patient was in the hospital for observation.

In June, 1946, the patient again developed a low-grade fever with general malaise. x-rays made by Dr. Wendell G. Scott revealed a shadow of the right upper lobe. His impression was that this was a questionable Ghon complex. X-rays made by Dr. Samuel B. Grant at a later date revealed that the lesion in the right upper lobe had practically disappeared and it was his impression that the patient had had a virus pneumonitis.

From September, 1946, until August, 1948, the patient had a few respiratory infections which left her with a low-grade fever over a long period of time. There was always an associated muscular pain. Various tests and x-rays revealed nothing abnormal. In August, 1948, she had a sudden onset of general malaise, nausea, and severe vertical headache. She

*Case report presented through the courtesy of Sim F. Beam, M.D.

also complained of diarrhea. Again, various tests including liver function, examination of the urine and blood, basal metabolic rate, x-rays of the chest, and a complete gastrointestinal series revealed no abnormalities.

Another respiratory infection developed in April, 1949. Sputum examination did not reveal pathogens. In June, 1949, in an effort to clear up the infection, the patient was given aureomycin orally, 0.25 Gm. over a period of seventy-two hours, without any improvement of symptoms.

At bronchoscopic examination performed on July 13, 1949, by Dr. James B. Costen, a yeastlike odor was noted. Specimens of the sputum taken at the time of the bronchoscopy were sent to the Barnard Free Skin and Cancer Hospital for mycologic examination.

Syringospora (Monilia, Candida) albicans was cultured from the sputum and the patient was accordingly placed on potassium iodide medication (saturated solution), 10 to 15 drops orally, three times daily. Six months of therapy resulted in remarkable clinical improvement with loss of fever. The patient is now working and well.

LABORATORY DATA

Sputum was streaked on several slants of Sabouraud's glucose agar. Within a few days there developed numerous colonies of pure culture of a yeastlike organism which was later identified as *Syringospora (Monilia, Candida) albicans.* About a week later, the cultures were again examined and found to have developed a golden yellow color somewhat suggestive of *Staphylococcus aureus.* Several colonies were examined microscopically and in the center of each colony there were found accumulations of golden yellow crystals (Fig. 1). With higher magnification it was noted that that portion of the growth adjacent to the crystalline material showed numerous large, thick-walled cells which are ordinarily considered to be chlamydospores and which are characteristic of *S. albicans* when grown on corn meal agar (Fig. 2).

Aureomycin hydrochloride when subjected to filtered ultraviolet radiation (Wood's light) shows a golden yellow fluorescence. When crystals of aureomycin hydrochloride are dropped on agar medium, they dissolve slowly, and as they do, the colored substance permeates the substratum. The amount and extent of permeation can be determined easily by the use of the Wood filter. Under Wood's light the original cultures with the yellow crystals in the center of each colony showed this yellow fluorescence with penetration into the adjacent medium. It seemed possible, therefore, that the substance might be either aureomycin hydrochloride or an altered form of the antibiotic.

Since it appeared that aureomycin hydrochloride or the changed crystals had an effect on the growth of *S. albicans,* it was decided to determine just how the effect took place. Accordingly, a number of plates containing Sabouraud's glucose agar were seeded with pure *S. albicans.* Immediately following this procedure, pure aureomycin hydrochloride* crystals were dropped in various patterns on the surface of the seeded agar plates. This was done in an effort to see whether the organism would develop and engulf the aureomycin in a manner similar to that noted in the sputum cultures. Within forty-eight hours, the growth of the organism was such that it covered the plate. The areas where the aureomycin crystals had been dropped, however, showed distinct inhibition of the organism's growth (Fig. 3). This appeared as a clear zone with an increasing amount of growth extending peripherally. When these plates were examined under Wood's light, a correlation of the inhibition could be made with the intensity of fluorescence. After several weeks of growth, the inhibited areas became less distinct. When examined again with Wood's light, the yellowish fluorescence could be seen throughout the whole plate.

It may be of special interest that the plates to which aureomycin had been added developed contaminants fairly rapidly in nearly all the plates, as compared with the controls

*Aureomycin hydrochloride, working standard, with a potency of 1,000 μg per milligram was graciously supplied by Dr. Henry Welch, Director, Division of Antibiotics, Federal Security Agency, Food and Drug Administration, Washington, D. C.

which remained uncontaminated. It is difficult to determine whether this was purely accidental, whether the presence of aureomycin stimulated the growth of chance contaminants, which is very likely, or whether the contaminating spores were present in the sample of aureomycin hydrochloride.

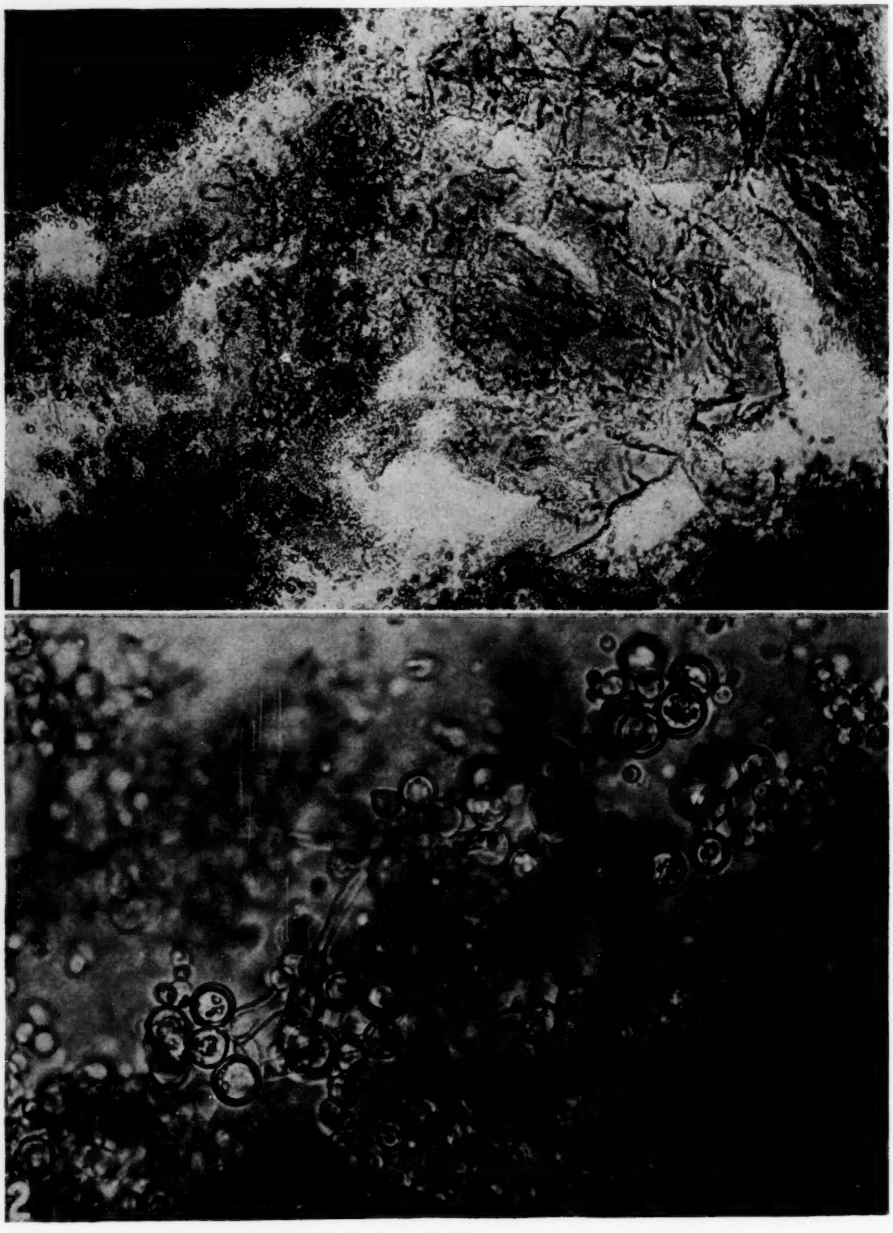

Fig. 1.—Amorphous aureomycin hydrochloride crystals or a variant in the center of a colony of *Syringospora albicans*, 101 days after inoculation. Unstained wet mount, ×105.

Fig. 2.—Organisms adjacent to the crystalline material showing large, round, thick-walled cells. Unstained wet mount, ×653.

Flasks containing 250 c.c. Sabouraud's glucose broth were inoculated with *S. albicans* and 50 mg. crystalline aureomycin hydrochloride. Growth as compared with the control appeared to be greater after seventy-two hours without any indication of inhibition. The clear broth developed a brownish color as compared with the control, because of the aureomycin. After several weeks of growth at room temperature, the amount of culture material at the bottom of the flasks appeared to be approximately twice the amount of that seen in the control flasks.

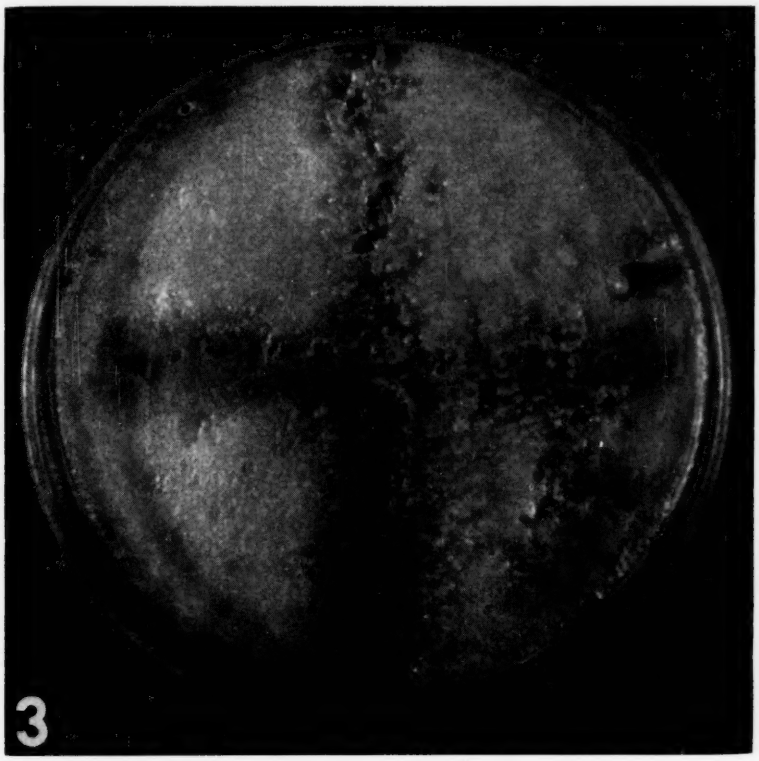

Fig. 3.—Culture of *Syringospora albicans* seeded with aureomycin hydrochloride, growing on Sabouraud's glucose agar, twenty-four days after inoculation. Note inhibition of growth along streaks where crystals were dropped.

Microscopic examination of the fungi in the aureomycin-containing flasks revealed some interesting information. Culture material obtained from the bottom of the flasks, in contact with the aureomycin crystals, showed numerous large cells (Figs. 4 and 5). These were approximately two to three times the size of those considered to be normal for the organism. They were seen scattered among the normal-sized cells as spherical to slightly ovoid, simple or budding cells (Fig. 4). In some flasks the organism could be seen in an attempt to form the pseudomycelium characteristic of the organism (Fig. 5). Here the cells were large and elongated, appearing somewhat in verticils or radiating in clusters, similar to what one sees with pseudomycelium arrangements. In scattered groups there could be seen clumps of two to four or sometimes more cells with thick walls simulating capsules (Fig. 6). These are probably to be considered as true chlamydospores and not to be confused with the large cells.

In contrast, a microscopic examination of the thin veil of growth on the surface of the broth showed chains of small yeastlike cells (Fig. 7). These were considerably smaller than the large cells and smaller also than the "normal" cells of *S. albicans*. They would appear to be rapidly growing and multiplying.

It is interesting to speculate on what effect aureomycin has on *S. albicans*. It is apparent that the antibiotic, like most of the known antibiotics, when present in concentrated form, may have a temporary inhibiting or fungistatic effect on the fungus. *S. albicans*, in general, is one of the least susceptible of the yeastlike organisms to such action. Whether this

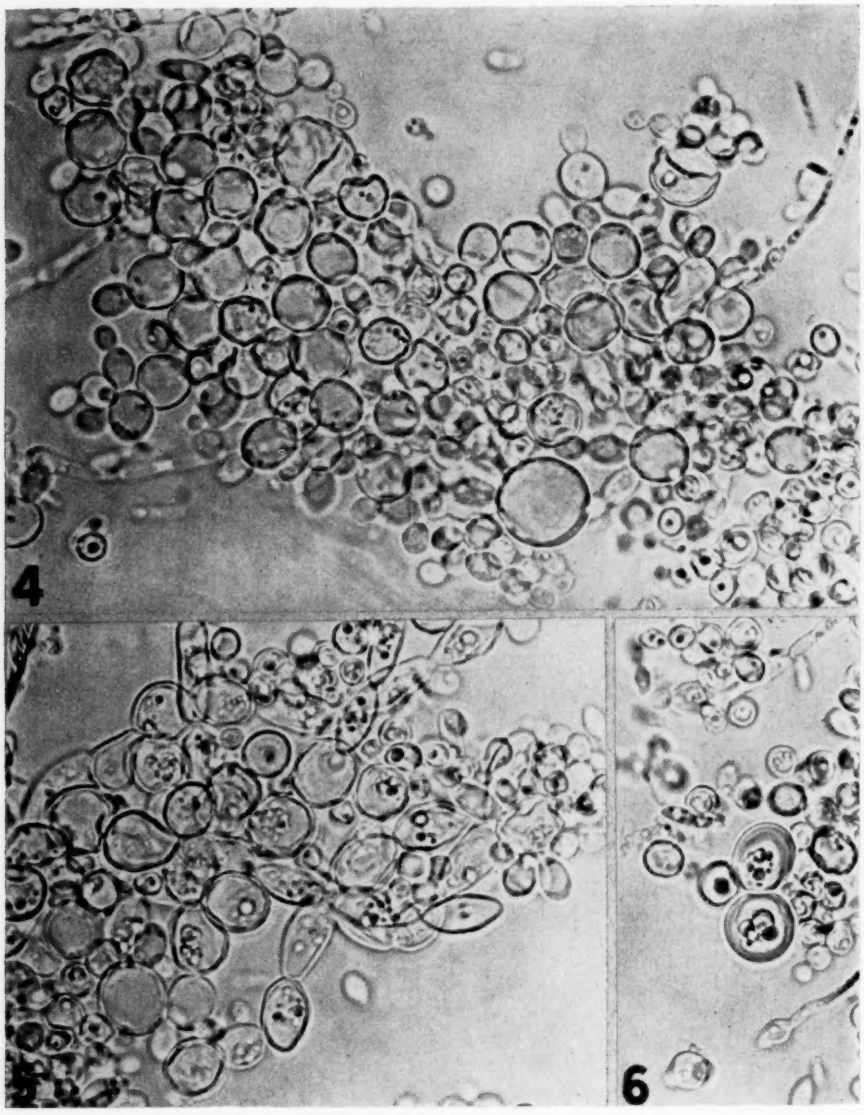

Fig. 4.—Large, round cells of *Syringospora albicans* growing close to aureomycin hydrochloride in Sabouraud's glucose broth. ×870.

Fig. 5.—Large, spherical to ovoid cells of *S. albicans*, in verticil arrangement, growing close to aureomycin hydrochloride in broth. ×870.

Fig. 6.—Spherical, thick-walled chlamydospores of *S. albicans* in broth. ×870.

action is due actually to antibiosis or possibly to a change in the immediate environment, such as a change in the hydrogen-ion concentration, thus affecting the physiologic behavior of the organism is something to be determined. In due course of time, the effect of the antibiotic crystals disappears, due chiefly to their instability in an aqueous medium and perhaps

in part to a diluting effect because of their dissolution in the substratum. The organism is a rapid grower and consequently will show good growth. It is possible that during the lag phase the fungus has built up a sufficient amount of internal energy in some form so that when external conditions become more favorable the cell will develop at a rate faster than that of the normal cell and occasionally produce a giant.

In a study of the effects of carcinogenic substances on *Saccharomyces ellipsoideus*, Dodge,[6] using methylcholanthrene in the medium, found that growth tended to be slower at the start than in the control. The control had a shorter lag but it was soon overtaken by the methylcholanthrene growth. "Except for occasional giant cells, the cells in methylcholanthrene are smaller and divide more frequently."

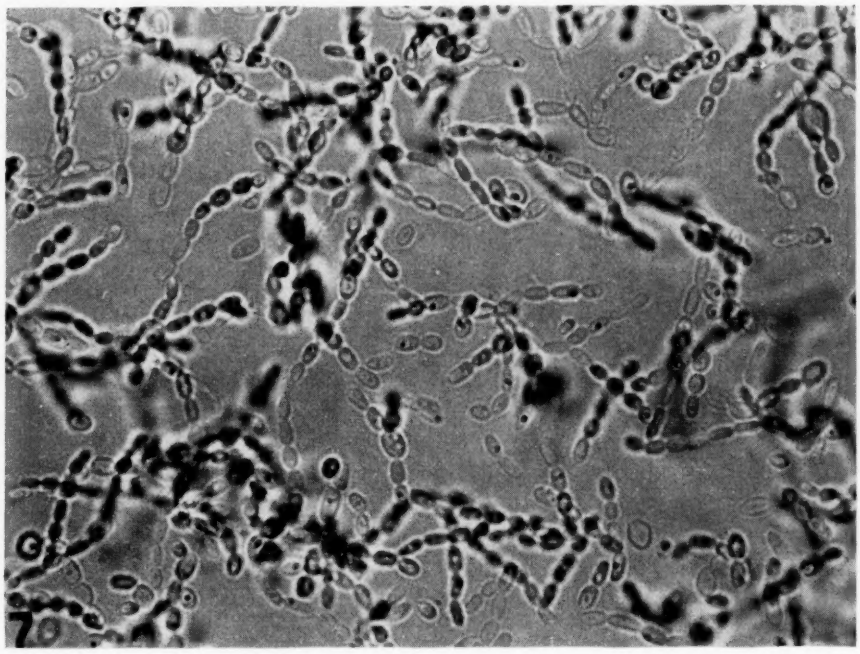

Fig. 7.—Small, rapidly multiplying cells in chains, from surface of broth medium containing aureomycin hydrochloride. ×788.

A comparison of the microscopic appearance of the cells of *S. ellipsoideus*, grown in media containing methylcholanthrene, and the cells of *S. albicans*, as grown in media containing aureomycin, indicates a similarity in response. In both cases we find large cells (giant cells), although the cells of *S. albicans* did not attain the size of those of *S. ellipsoideus* as reported by Dodge, and the small, frequently dividing cells. Whether aureomycin has carcinogenic capabilities is something that requires investigation.

DISCUSSION

Toxic or side effects from the administration of therapeutic agents are not unusual. The sulfonamides were notorious for the numerous and in some cases serious complications to which the patient was subjected. The advent of the antibiotics similarly produced many and varied reactions. Much has been written concerning these toxic phenomena when penicillin was given orally. Clinically, many of the side effects listed in this paper have been found to occur

also when antibiotics other than aureomycin were administered.[7] The patients complained of looseness of stools or diarrhea and pruritus ani which often is an accompanying feature in diarrhea when antibiotics such as polymyxin, chloramphenicol, and terramycin, to name a few, are taken. In most instances, antibiotics when given orally affect the intestinal flora most remarkably either by lysis or by inhibition of the bacteria so that it is relatively easy to culture pure yeastlike organisms.

It is a little difficult to state emphatically whether the gastrointestinal disturbances are due to the high acidity of the gastrointestinal tract with the destruction of the normal intestinal flora as Harris presumed or to the resultant increase in growth of the yeastlike organisms. It is also difficult to designate allergy per se as specifically responsible for the mucosal and dermal reactions. Certainly the altered physiology of the gastrointestinal tract as a result of the elimination of the normal flora may account for these manifestations and the application of the term allergy to such a phenomenon is debatable. Oral and gastrointestinal disturbances and the cultivation in large numbers of yeastlike organisms are not uncommonly found in tropical sprue. In sprue, however, gastric analysis reveals hypoacidity although the stools are acid because of unabsorbed fatty acids. Furthermore, many patients taking brewers' yeast for therapeutic reasons often complain of diarrhea. Consequently, one must consider the excessive amount of yeastlike organisms as a likely cause of the gastrointestinal upset, at least in some cases.

Oral lesions following aureomycin intake have been designated as sensitivity to the drug. The clinical appearance of these lesions in many ways simulates that of moniliasis. The finding of yeastlike organisms in the mucous membrane lesions of the mouth, throat, pharynx, and in the perlèche-like lesions, especially in female patients, seems to indicate rather strongly, at least in some patients, infection with these organisms as a consequence of the aureomycin. The similarity to sprue is also great, so that one should consider the possibility that the gastrointestinal upset may interfere with the nutritional requirements and consequently give rise to the oral symptoms. The same apparently holds for the patients who develop vaginitis. The finding of large amounts of yeastlike cells again would tend to suggest that these organisms, whether stimulated by the aureomycin or allowed to grow in abundance because of the action of aureomycin in eliminating bacteria, play a major role in the production of such lesions. Especially is this of significance when it is seen that such patients in many instances develop the characteristic satellite lesions on the adjacent thighs.

To further emphasize this point, we need only to re-examine the in vitro action of aureomycin on *S. albicans:* It is apparent that the antibiotic, in a concentration of 0.2 mg. per cubic centimeter, had a stimulatory effect on this organism, producing not only large cells and rapidly multiplying cells but also an increased over-all production of cells as compared with the control. This should be considered very significant.

An evaluation of the data assembled in this paper clearly indicates that in the administration of aureomycin hydrochloride the clinician should expect to

find, at least in women, a good percentage of whom are carriers of yeastlike organisms, complicating lesions due to these organisms. More specifically, however, the clinician should rule out bronchial or pulmonary moniliasis, by culture preferably, before prescribing oral aureomycin for extended periods. The data and the case presented here make it clear that oral aureomycin is contraindicated in patients with such lesions. As a matter of fact, it would be unwise to prescribe any of the newer antibiotics in such cases before first determining their effect on *S. albicans*. There is a final consideration which concerns those cases in which aureomycin is prescribed for diseases of established etiology, other than moniliasis, and in which the patient develops monilial or monilial-like lesions. In these cases it may be advisable to administer a drug, such as potassium iodide, which is of known value in combating moniliasis, along with the antibiotic, provided the iodide does not adversely affect the primary lesions. The iodide should control, in a great measure, the yeastlike secondary involvement.

SUMMARY

The toxic effects following the oral administration of aureomycin hydrochloride are listed as nausea, vomiting, headache, epigastric pain, persistent diarrhea, a maculopapular, urticarial, or scarlatiniform skin rash, anal and perineal irritation and/or pruritus, vaginitis, lesions of the mucous membranes of the mouth, throat, and pharynx, perlèche-like lesions, black tongue, glossitis, cheilitis, and smooth tongue. Phlebitis has been described as a sequela to intravenous administration of aureomycin.

A patient, with a complicated history of pulmonary lesions, from whose sputum *Syringospora (Monilia, Candida) albicans* was isolated following oral aureomycin therapy is described and presented. Golden yellow crystals considered to be possibly an altered form of aureomycin hydrochloride were found in the center of each monilial colony.

Aureomycin crystals in concentrated form when placed on plates seeded with *S. albicans* tend to show temporary inhibition of the organism's growth.

Dilute aureomycin has a stimulatory effect on *S. albicans*, increasing the perceptible growth of the culture as compared with the control.

Microscopically, aureomycin has a double action. Cells closely associated with the aureomycin develop large or giant forms whereas cells some distance from the crystals but nevertheless in contact with diluted aureomycin develop small, rapidly multiplying forms.

Aureomycin hydrochloride fluoresces bright golden yellow and this was demonstrated in the primarily isolated colonies of Syringospora.

Oral aureomycin is contraindicated in pulmonary mycosis caused by *S. albicans*.

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NOCARDIA ASTEROIDES: STUDIES OF ITS PATHOGENICITY AND DRUG SENSITIVITIES

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LABORATORY investigations of drug sensitivities and pathogenicity of the aerobic actinomycete *Nocardia asteroides* have been few and conflicting. No report of experimental in vivo drug testing has been found in the literature. The purpose of the present investigation was to determine the in vivo as well as in vitro drug sensitities of some strains of *N. asteroides*, and to establish a procedure for determination of pathogenicity.

While sulfadiazine has been thought to have been efficacious in clinical cases of nocardiosis,[1-4] one strain of *N. asteroides* has been reported to be quite insensitive to sulfadiazine.[5] Streptomycin was reported to be effective in vitro[6] but was not found to be successful in the treatment of a human patient.[1] An isolated mention of sensitivity to aureomycin[5] and a report that Nocardia is resistant to terramycin[7] are in the literature. Penicillin, although widely used for nocardiosis, is definitely not well established as a successful drug for this disease.[1, 8, 9]

Determination of pathogenicity is of primary importance because saprophytic actinomycetes are among the commonest of laboratory contaminants. Gordon and Hagan[10] isolated from soil or potatoes 130 strains of Nocardia, some of which were pathogenic. The important observation made was that the pathogens were indistinguishable from the nonpathogens on the basis of cultural or fermentation characteristics. The literature abounds in conflicting reports as to the pathogenicity of *N. asteroides* for guinea pigs and rabbits.[11] Mice seem to have been used by few investigators[3, 13-17] and have been stated to be unsuitable for pathogenicity testing.[18] As shown in the present investigation, however, mice may successfully be used and, indeed, are superior to other animals in some respects.

MATERIALS AND METHODS

The strains of Nocardia employed were as follows: two strains recently isolated from Lawson VA Hospital patients, G and E; a relatively old laboratory strain, A; two strains of dubious pathogenicity, W (isolated from gastric specimen), and 267, a Nocardia-like actinomycete of uncertain identity.* Working cultures were maintained in flasks of proteose-peptone-dextrose broth, pH 7, at 37° C. Young (5-day-old) mycelial mats were homogenized by shaking for five minutes with glass beads in a Florence flask on a Kahn shaker. Sterile saline was added gradually and the suspension transferred to a tube for settling of larger particles. After twenty to thirty minutes the supernatant was removed to another tube, and

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an aliquot taken for the determination of concentration by centrifugation in a Hopkins tube. The remainder of the supernatant was then diluted to the desired concentration. For in vivo experiments inocula of from 4 to 30 mg. per milliliter were employed. These were prepared on the assumption that 1 c.mm. of packed organisms is equivalent to 1 milligram.

For the in vitro tests the serial dilution technique was employed, using the proteose-peptone-dextrose broth at pH 7. Each tube containing 5 ml. of medium was seeded with 0.1 ml. of a suspension containing the equivalent of 0.15 c.mm. of packed cells per milliliter. This inoculum was found to provide first appearance of surface growth in control tubes on the second day after planting.

Drugs from commercial sources were freshly prepared for each experiment. If not sterile they were Seitz-filtered. Usually four tubes of each concentration were employed. Each test was confirmed by one or more repetitions. Cultures were incubated at 37° C. and examined each day.

RESULTS

Pathogenicity Tests.—Preliminary tests indicated that in spite of reports to the contrary,[11, 18] our strains of Nocardia were pathogenic for mice. The notorious variability in response of individual animals to nocardial infection, making important the use of several animals for any test, indicated the desirability of establishing a reliable procedure for virulence testing in mice. Tests were made to establish the amount of inoculum required for definite pathogenesis. While intravenous and intracerebral routes of inoculation proved to be successful, the intraperitoneal route was found to be most practical because of its relative simplicity and applicability to mice of all sizes. White mice in one test were demonstrated to be susceptible to infection; dba mice were used in all other tests. A summary of results is given in Table I.

With the inocula employed its is evident that more mice will survive than will die of the infection. Mice surviving five days were usually found to recover from the infection. However, the formation of lesions three to six days after infection is invariable, and sacrifice for autopsy during this period is therefore indicated. The number, size, and extent of lesions are greater after five days than after two or three. Lesions in mice correspond to those that occur in guinea pigs or rabbits[12]: a diffuse peritonitis with numerous whitish 1 to 7 mm. nodules in omentum, lower diaphragm, and variably in spleen, liver, lungs, heart, kidney, et cetera. Smears of nodules almost invariably reveal the slightly acid-fast pathogen in the form of branching hyphae of various lengths, including many bacillary fragments. Sometimes tangled masses of the organism

TABLE I. MOUSE DEATHS DURING A PERIOD OF FIVE DAYS FOLLOWING INTRAPERITONEAL INOCULATION WITH N. ASTEROIDES IN PHYSIOLOGIC SALINE

STRAIN	INOCULUM (MG.)	NUMBER OF MICE	PER CENT MORTALITY—5 DAYS
G	14	10	60
	10	10	30
	5	5	0
	4	20	20
	2	20	10
E	10	10	30
	5	30	15
A	10	20	0
	8	10	30

occur which might be described as microscopic granules. Pure cultures of *N. asteroides* are readily obtained from the pathologic mouse tissues. After ten days or more most mice will have extensive lesions, often with adhesions between the various abdominal organs and the peritoneum, while a few mice show little evidence of infection. Microscopic study of histologic preparations of the organs of the infected mice showed usually very acute small multiple abscesses both on the serosal surfaces and to a lesser extent inside the organ involved. The usual abscess is composed of mycelium surrounded by necrotic material and polymorphonuclear leucocytes, with a variable amount of granulomatous reaction.

Some evidence of decreasing virulence with continued laboratory culture was seen. The same inoculum did not always give the same proportion of deaths in five days. This was especially evident in the case of the laboratory strain A, which was clearly less virulent than the freshly isolated strains G and E.

Inoculation in mucin instead of saline suspension resulted in a much higher percentage mortality (Table II). Wilson hog mucin (Type 1701-W) was emulsified to 4 per cent in water by use of a Waring Blendor; the product was autoclaved and after cooling was adjusted to pH 7.3 with sterile NaOH. After sterility test the preparation was stored in the refrigerator. Sedimented Nocardia was suspended in this emulsion for inoculation of mice. Control mice injected with mucin alone or with mucin plus the saprophyte *N. opaca* remained evidently healthy. One hundred per cent of the test mice were killed by injection of as little as 3 mg. of strains G, E, or A. Mice inoculated with strain A were somewhat slower to die than those inoculated with strains G and E, in this way exhibiting the lower virulence that was also indicated by strain A in the tests made without mucin (Table I).

Strains 267 and W were reported to have been isolated from human patients, but their etiologic relation to disease was doubtful. As tested with mucin their virulence was shown to be much less than that of the strains G, E, and A. However, W was shown to have definite if low pathogenicity for mice, and 267

TABLE II. N. ASTEROIDES SUSPENDED IN 4 PER CENT MUCIN: MOUSE DEATHS FOLLOWING INTRAPERITONEAL INOCULATION

STRAIN	INOCULUM (MG.)	NUMBER OF MICE	PER CENT MORTALITY—5 DAYS
G	10	5	100
	5	10	100
	3	5	100
E	5	20	100
	3	10	100
	2	20	100
	1.5	15	60
	1	10	60
A	10	5	100
	5	5	80*
	3	5	60*
W	10	5	20†
267	10	5	0

*100 per cent mortality by tenth day.
†40 per cent mortality by eighth day.

produced lesions of the same type as the more pathogenic strains. Mice inoculated with 10 mg. of strain W or 267 without mucin likewise showed these lesions but survived the five-day period without fatalities.

In Vitro Drug Tests.—A summary of results of the in vitro tests with the three strains of *N. asteroides* G, E, and A is given in Table III. The three strains exhibited an impressive similarity in sensitivity to these drugs. It is evident that aureomycin, streptomycin, and sulfadiazine are inhibitory of growth in low and approximately equal concentrations, while terramycin, chloromycetin, and especially penicillin and para-aminosalicylic acid are relatively ineffective. Aureomycin is outstanding in producing complete inhibition of growth in the concentrations stated, while sulfadiazine permits a certain small amount of growth within twenty-four hours in every concentration tested (up to 600 micrograms per milliliter). However, the effectiveness of sulfadiazine cannot be doubted. At a time when a heavy pellicle has formed in control tubes, no surface growth is present in sulfadiazine-containing tubes.

TABLE III. MINIMUM CONCENTRATIONS OF DRUGS COMPLETELY INHIBITING SURFACE GROWTH OF N. ASTEROIDES IN SABOURAUD DEXTROSE BROTH, PH 7

DRUGS	48 HOURS	72 HOURS	10 OR MORE DAYS
Aureomycin	4 μg/ml.	16 μg/ml.	128 μg/ml.
Streptomycin	4 μg/ml.	16 μg/ml.	128 μg/ml.
Sulfadiazine	4 μg/ml.	16 μg/ml.	128 μg/ml.
Terramycin	16 μg/ml.	32 μg/ml.	—
Chloromycetin	25 μg/ml.	75 μg/ml.	200 μg/ml.
Penicillin	500 μg/ml.	1,000 μg/ml.	—
Para-aminosalicylic acid	5 mg./ml.	10 mg./ml.	—

The anomalous development of Nocardia as a sediment during the first twelve to eighteen hours irrespective of sulfadiazine concentration was puzzling. That it was not due to the special conditions on the surface as compared to those in the body of the liquid was shown by a number of tests. Inocula carefully floated on the surface showed the same initial growth on the surface as had the usual submerged inocula below the surface. Tests made on sulfadiazine-containing agar revealed the same phenomena as seen in broth: growth at first irrespective of drug concentration on the surface of the agar (evident with a microscope), then inhibition depending on drug concentration.

It was observed that inocula from the twenty-four-hour growth in sulfadiazine-containing tubes when transferred to other sulfadiazine tubes developed very slowly or not at all, indicating perhaps that some metabolite present in the inoculum, inaccessible to the action of sulfadiazine, was adequate for the initial growth.

The eventual development of organisms in drug concentrations initially inhibitory is a familiar phenomenon commonly due to deterioration of the drug or to the appearance of a resistant population of organisms. Tests demonstrated that no development of resistance to sulfadiazine occurred, and sulfadiazine in sterile Sabouraud medium was found to be perfectly stable at incubator temperature for the time span of these experiments. But that sulfadiazine was

not stable in the presence of Nocardia was indicated by reinoculation of sulfadiazine medium from which initially appearing growth had been removed by Seitz-filtration; growth then appeared as if no drug was present. Indeed, sulfadiazine added to such filtrates was not effective in checking nocardial growth, suggesting that an excess of some antagonist to sulfadiazine had originally been formed in the medium.

Para-aminobenzoic acid was found to antagonize the effect of sulfadiazine on Nocardia, as has been found for most sulfadiazine-resistant bacterial species.

In Vivo Drug Tests.—Methods: Intraperitoneal inoculations of mice were made as described previously and were given in quantity judged sufficient to result in a high but less than 100 per cent mortality. Drugs were administered subcutaneously or, in later experiments, intramuscularly. For most drugs one injection per day was administered. After six days or longer surviving mice were sacrificed and autopsied. The degree of pathologic change was estimated by the number and size of lesions at the site of inoculation, omentum, spleen, liver, and diaphragm.

Penicillin: Although our strains of Nocardia were insensitive to penicillin in vitro, it was thought worth while to make an in vivo test since some reports[5, 21] have indicated that improvement has occurred in patients treated with penicillin. Ten mice were inoculated with 2 mg. of strain E in mucin; five of these mice were given daily subcutaneous injections of 5 mg. (8,000 units) of Schenley penicillin G. Within five days, 60 per cent of the control mice and all of the treated mice were dead.

Aureomycin: Because of the promising indications of in vitro testing with aureomycin several trials of this drug were made in vivo. In the first test, 2 mg. were injected subcutaneously each day, and the drug was mixed with the food to the extent of 1 per cent. Two of twenty mice so treated succumbed to the infection as compared with seven of the control group. In two subsequent tests, increased dosage (3 or 5 mg.) evidently afforded no protection. Toxicity of the drug as administered was thought to be interfering with the latter tests. In the next experiment, therefore, the drug was given by stomach tube, 5 mg. twice a day. Again there was no evidence that aureomycin was effective: all of both treated and control mice died of the infection. Subsequently, note was made of the recommendation of the intramuscular route for administration of aureomycin to mice.[19] Each of fifteen mice was given 3 mg. intramuscularly on the day of inoculation and 2 mg. on each of the next five days. No effect due to aureomycin was evident on the basis of gross pathology of mice surviving on the sixth day. However, only two of the aureomycin-treated mice died, while eight of fifteen control mice died of nocardiosis.

Streptomycin: Twenty of forty mice inoculated with *N. asteroides* were treated with streptomycin. Ten of these received 3 mg. a day subcutaneously and ten received 5 mg. a day. Twelve of twenty control mice and eleven of the treated mice died of the infection.

Sulfadiazine: Fairly consistent and definite results were obtained with sulfadiazine (Table IV). Except for the third experiment where injections

TABLE IV. SULFADIAZINE TREATMENT OF NOCARDIOSIS IN MICE

EXPERIMENT	DOSAGE PER DAY (MG.)	ROUTE	PER CENT MORTALITY		NUMBER OF MICE
			TREATED	CONTROL	
1	5	Subcu.	30	90	20
2	5	Subcu.	20	60	10
3	5	Subcu.	90	100	20
4	5	I.M.	0	50	30
Total average			30	75	80

evidently were not started early enough to stem the overwhelming infection (all control mice were dead in forty-eight hours), there was impressively lower mortality for the treated than for the control mice. In the first and fourth experiments the degree of pathologic change of control mice surviving eight days was approximately three times that of the mice treated with sulfadiazine.

DISCUSSION

A method of easy application and dependable accuracy for virulence testing is suggested by the data presented. The inoculum indicated is ½ ml. of a suspension of young homogenized growth adjusted to 20 mg.—i.e. 20 c.mm. of centrifuge-packed cells—per milliliter. With such an inoculum probably fewer mice will die than survive in five days, but at this time all will show the typical nodules on peritoneal membranes, if the strain is pathogenic. With mucin such a strain in even smaller dosage may be expected to produce prompt death of all mice injected with it. Differences in degree of virulence may be estimated from the proportion of mice surviving five days after inoculation of a mucin suspension of the organism.

It was learned after the completion of the present study that Strauss and Kligman[20] have independently shown that mucin furthers the pathogenic process of nocardiosis in mice. Their results are similar to ours, but evidently their strain was less virulent than our strains G and E.

The multiplicity of morphologic and physiologic types of aerobic actinomycetes suggests that there may be strain difference in drug sensitivities. In the present investigations no appreciable difference was found in the drug sensitivities of the three strains tested. Hager, Miggliaccio, and Young,[5] however, reported one of their two strains of *N. asteroides* to be insensitive to sulfadiazine. Furthermore, in contrast to other workers, they found both of their strains sensitive to penicillin. The laboratory work of these investigators was only very briefly reported, but if confirmed it re-emphasizes the practical importance of drug sensitivity tests for determination of the most promising chemotherapy for human treatment.

In determination of drug sensitivity in liquid media it is important to recognize the two phases of growth: the early (submerged) growth and the later-appearing pellicle. If pellicle formation were the only criterion used for determination of drug sensitivity, the difference between aureomycin and sulfadiazine would not be seen; if, on the other hand, readings were made only at the end of twenty-four hours, sulfadiazine would appear to be quite ineffective.

The importance of in vivo drug testing, evidently not heretofore done with Nocardia, is shown in the lack of parallelism with in vitro studies. Our in vivo tests were in accord with those done in vitro with sulfadiazine and penicillin, but not for streptomycin. Most of the in vivo tests of aureomycin failed to show benefit that might have been expected from the in vitro tests.

SUMMARY

1. Two strains of *Nocardia asteroides* recently isolated from pulmonary infections of man and a laboratory strain of this organism were found to have similar drug sensitivities but differed in virulence.

2. Methods of standardization of inocula are described.

3. Mice are susceptible to nocardiosis and suitable for virulence testing.

4. Addition of mucin to the inoculum increases the mortality rate.

5. Mice treated with streptomycin were not protected, although this drug was inhibitory in vitro.

6. Aureomycin, most inhibitory of any of the drugs tested in vitro, was doubtfully effective in mice.

7. Penicillin was ineffective in vivo as well as in vitro.

8. Sulfadiazine checked the development of Nocardia in the test tube and decreased the mortality rate of mice infected with this pathogen.

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THE REACTION OF HUMAN SERUM AND COMPONENT PROTEINS OF HUMAN PLASMA WITH A QUATERNARY AMMONIUM SALT, OCTAB

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QUATERNARY ammonium compounds such as octadecyl dimethyl benzyl ammonium chloride (Octab)* and cetyl dimethyl benzyl ammonium chloride (Cetylon) combine with hyaluronic acid or desoxyribonucleic acid to form turbid solutions or precipitates.[1] Since the degree of turbidity is proportional to the concentration of hyaluronic or desoxyribonucleic acid, an attempt was made to apply the reaction between hyaluronic acid and the quaternary ammonium salt to an assay technique for the determination of streptococcal antihyaluronidase activity in serum.[1] During this investigation it was found that certain serum proteins also give a turbidity with the quaternary ammonium compounds, and that in patients with acute rheumatic fever the slight amount of turbidity contributed by the serum fraction was not always constant. Further investigation revealed that the degree of turbidity of serum proteins was uniformly increased in acute phase sera in contrast to normal sera. This report is concerned with the reaction of serum with quaternary ammonium compounds.

EXPERIMENTAL METHOD

The measurement of the turbidity of human serum with quaternary ammonium compounds is made in the following manner. Human serum obtained by separation of serum from whole blood clotted in 10 by 1.5 cm. sterile glass tubes is diluted 1:100 in 0.08M sodium chloride solution. A 2 ml. aliquot of diluted serum, corresponding to 0.02 ml. of whole serum or approximately 1.2 mg. of serum protein, is pipetted into a 15 ml. cuvette. To this are added 5 ml. of 0.08M sodium chloride buffered to pH 6.8 by 0.05M collidine buffer.[2] One milliliter of 0.1 per cent Octab in distilled water is then added, and the contents of the tube are mixed. A turbidity develops immediately which increases in amount during a period of twenty minutes. The percentage transmission of light is read in a Kromatrol photometer (using filter 460) after maximum turbidity has developed. The turbidity unit is then expressed as $\log_{10}\frac{I_0}{I} \times 1{,}000$.†

RESULTS

Variations of Turbidity in Sera of Normal and Abnormal Individuals.—The sera of ninety-seven normal medical students were assayed for turbidity. The distribution of turbidity values for this group is shown in Fig. 1. It will be observed that all sera cause a turbidity greater than 50 units, with most values lying between 70 and 100 units. Whenever values greater than 100 are

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*A variety of quaternary ammonium compounds including octadecyl dimethyl benzyl ammonium chloride (Octab) was supplied through the generosity of Mr. Wellington Rounds, Fairfield Laboratories, Inc., Plainfield, N. J.

†A barium sulfate standard prepared by addition of 3.0 ml. of 1 per cent $BaCl_2.2H_2O$ to 100 ml. of 0.2N H_2SO_4 gave an optical density of 0.205. Comparable values are obtained with a Coleman Junior photometer.

encountered, there is usually some infectious process to be found in the patient. For example, respiratory infections in otherwise normal subjects cause an increase of serum turbidity to values between 120 and 130 units.

Individuals who have a severe illness due to such unrelated pathologic changes as carcinoma, pneumonia, rheumatic fever, and rheumatoid arthritis also have greatly increased turbidity indices, in some instances as high as 260 units. In another communication[3] it is shown that the determination of the Octab turbidity index is a reliable and convenient method for estimating inflammatory activity during the course of rheumatic fever.

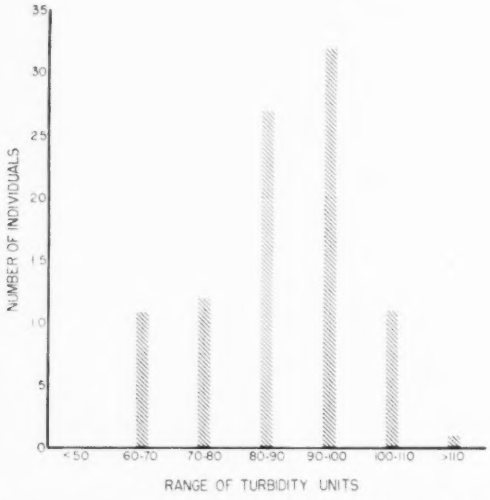

Fig. 1.—Distribution of serum-Octab turbidity values in a group of normal medical students.

Relationship of Octab Turbidity in Serum to Other Abnormal Components of Acute Phase Sera.—Various procedures were carried out in an attempt to characterize the substance in serum which gives a turbidity with quaternary ammonium compounds. In each instance the treated sera were tested in the same manner as already described. It was found that dialysis of serum in a cellophane membrane for five days against running tap water produced no loss of the serum component causing turbidity upon addition of Octab. When diluted serum was heated for fifteen minutes at 60° C., no change in turbidity resulting from adding Octab was noted. Prolonged storage at 4° C. caused no change other than a slight increase in turbidity which may be related to concentration of serum by evaporation.

Adsorption of acute phase serum with Type 27 pneumococci to remove the C-reactive protein[4] produced no decrease in serum turbidity given with Octab, nor was any alteration of turbidity observed after treating either normal or acute phase sera with *Bacillus subtilis*.[5]

Reports of an elevated serum mucoprotein in various infections suggested that this might be the protein component reacting with Octab.[6] Serum

mucoprotein was prepared by Winzler's method.[6] When this protein was added to Octab, even in a hundredfold concentration above maximum increments found in pathologic sera, no turbidity was formed.

Correlative studies were made to compare the serum inhibition for testicular hyaluronidase[7] with the degree of serum turbidity given with Octab. In general, while each was elevated in acute infectious processes, no proportionality of one to the other could be established. This suggests that the hyaluronidase-inhibitor factor differs from the turbidity-producing substance. The heat stability of the latter also precludes similarity since the hyaluronidase inhibitor is heat labile.

Crude fractionation of acute phase sera by means of $(NH_4)_2SO_4$ precipitation has been carried out. After dialysis in a cellophane tube in running tap water for two days, the albumin and globulin fractions were reconstituted in 0.85 per cent sodium chloride solution to the same protein concentration as the original serum. The component which reacts with Octab resides in the globulin fraction.

Effect of Salt Concentration and pH on Serum Turbidity.—The reaction of an anionic detergent, sodium lauryl sulfate, with various proteins is influenced by salt concentration, pH, and concentration of reactants.[8] Only a few similar observations have been made with respect to cationic detergents of the quaternary ammonium type. Tice and Pressman[9] observed that "anionic gelatin" formed turbidity with Zephirol (a mixture of alkyl dimethyl ammonium chlorides) at pH 6.0 but not at pH 4.0. "Cationic gelatin" did not produce turbidity with Zephirol at any pH. Jaffé[10] showed that addition of Zephirol to protein solutions of 10 mg. per milliliter concentration caused precipitation at the isoelectric point of several different proteins studied.

In order to study the effect of variation of pH on the reaction of Octab with serum and two component proteins of serum, a series of 0.05M buffers were prepared varying from pH 5.0 to 10.5. Acetic acid-sodium acetate buffers at pH 5.0 to 5.5, KH_2PO_4-Na_2HPO_4 buffers of pH 6.0 to 7.5, boric acid-sodium borate buffers of pH 8.0 to 9.5, and glycine-sodium glycine buffers of pH 10.0 to 10.5 were used. The pH of each buffer was determined by glass electrode measurement.

Human albumin,* gamma globulin,† and whole serum were diluted in 0.08M sodium chloride solution to a concentration of 0.6 mg. per milliliter. Two milliliter aliquots were then added to 5 ml. of the various buffers, and turbidity was determined after addition of 1.0 ml. of 0.1 per cent Octab.

The results of this experiment are illustrated in Fig. 2. It will be observed that albumin produces no turbidity with Octab until pH 9.0 is reached. Gamma globulin causes slight turbidity at pH 7.0. In contrast, serum causes turbidity to form at pH 5.5 which increases progressively with increase in pH. It will

*Concentrated (25 Gm. per 100 ml.) salt-poor human albumin was obtained from E. R. Squibb & Sons, New York.

†Immune serum globulin (human) in concentration of 160 mg. per milliliter was obtained from Cutter Laboratories, Berkeley, Calif.

be noted, however, that the increase in serum turbidity in the pH range of 6.5 to 7.5 occurred only when a collidine buffer was used. A variation of the pH of the phosphate buffer in this range caused no change in serum turbidity after addition of Octab. Collidine buffers also appear to enhance the reactivity of albumin while that of gamma globulin is unchanged. These data suggest

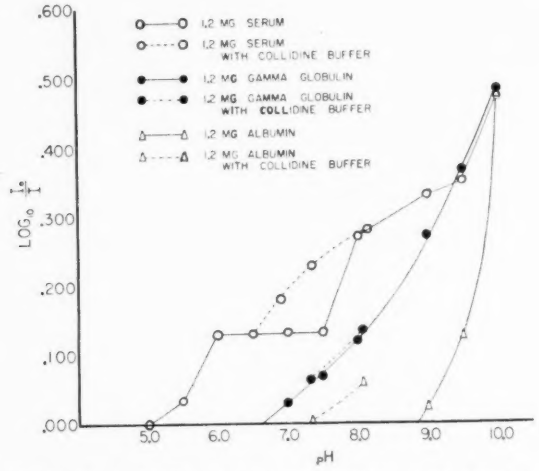

Fig. 2.—Effect of pH on turbidity of various proteins with Octab. Equivalent amounts of the different proteins tested were added to an equimolar salt system which was varied only in respect to pH. The optical density resulting from addition of Octab is plotted against variation in pH.

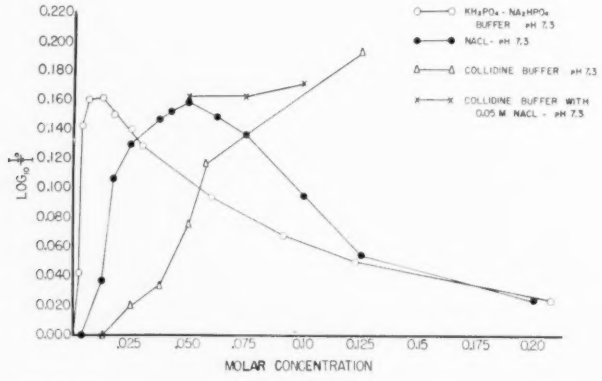

Fig. 3.—The effect of salt concentration on turbidity of serum proteins with Octab. By means of the indicated buffers (at constant pH of 7.3), increasing concentrations of buffer were added to 0.02 ml. of human serum. The turbidity resulting from addition of constant amounts of Octab was then expressed as optical density and plotted against the molar concentration of the various salts.

that phosphate may inhibit reactivity of some of the serum proteins with Octab in a pH range of 7.0 to 8.0. The data also indicate that the reacting component of serum in pH range of 5.5 to 7.0 is neither albumin nor gamma globulin.

The effect of salt concentration on reactivity of serum with Octab is shown in Fig. 3. Collidine and Na_2HPO_4-KH_2PO_4 buffers at pH 7.3 were

diluted suitably to make solutions of decreasing molar concentration. A sodium chloride solution adjusted to pH 7.3 with NaOH was similarly employed in decreasing molar concentrations. It will be observed that an optimal salt concentration is required for maximal turbidity formation. In the case of phosphate buffer a concentration of 0.0125M is optimal, whereas with NaCl the optimal concentration is 0.05M. An increase in concentration of either sodium chloride or phosphate beyond these limits inhibits the turbidity reaction of Octab with serum. Conversely, collidine buffer in increasing concentrations causes increasing turbidity. If collidine buffer is prepared in 0.08M sodium chloride solution, variations in salt concentration have less effect on turbidity.

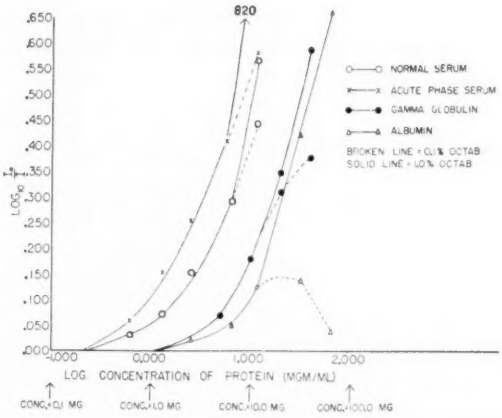

Fig. 4.—Effect of protein concentration on Octab turbidity. Increasing amounts of the indicated proteins were added to a system containing 0.08M sodium chloride buffered with 0.05M collidine at pH 6.8. The turbidity resulting from addition of either 0.1 per cent or 1.0 per cent Octab was then plotted as optical density against the $\log_{10}$ concentration of protein (milligrams per milliliter).

The effect of concentration of the reaction components in the protein-quaternary ammonium system was also studied. In this experiment varying concentrations of albumin, gamma globulin, and normal or acute phase serum were made up in 0.08M sodium chloride solution buffered to pH 6.8 with 0.05M collidine buffer. A solution of 0.1 per cent Octab was added to one series of protein dilutions, and a 1.0 per cent Octab solution was added to another series of dilutions. Inspection of Fig. 4 reveals that normal serum produces an optical density of 0.165 at 3.2 mg. per milliliter concentration. Whereas an acute phase serum from an individual with rheumatic fever gives an optical density of 0.285 with the same total protein concentrations. In this same concentration, albumin and gamma globulin with Octab give optical densities of 0.025 and 0.040 respectively. It is thus clear that the albumin and gamma globulin fractions contribute very little to the turbidity given by whole serum. Only after albumin reaches a concentration of 18 mg. per milliliter and gamma globulin 10 mg. per milliliter does one approximate the turbidity value given with 3.2 mg. per milliliter of whole serum.

The reaction of Octab with albumin, as shown in Fig. 4, is quantitatively different from that of Octab with serum or gamma globulin in that a marked decrease in turbidity occurs when 0.1 per cent Octab is added to albumin solutions of increasing concentrations. With serum or gamma globulin the decrease in turbidity with 0.1 per cent Octab as the protein concentration increases is much less marked. These results at present are not easily explainable.

DISCUSSION

The interaction of proteins and synthetic detergents may be related to electrostatic attractions between oppositely charged ionic groups and to an association of nonpolar residues. The effect of this reaction may be manifested by precipitation, by denaturation, or even by inhibition of heat coagulation reactions. Although synthetic detergents inhibit the coagulation of denatured proteins, the same detergents precipitate native proteins under certain conditions of pH and of weight ratio of protein to detergent.[11] In the case of anionic detergents, the amount of detergent bound is stoichiometrically related to the number of basic groups in the protein.[11]

The demonstration of Tice and Pressman[9] that "anionic" but not "cationic" gelatin reacts with a quaternary ammonium salt, Zephirol, to form a precipitate suggests that, conversely, the reaction of protein with cationic detergents is related to the acidic groups in the protein.

That the reaction of human serum with a quaternary ammonium salt is distinctly different from the reaction of albumin and gamma globulin is evident from a study of the effect of pH and the ratio of detergent to protein. In the former instance turbidity forms with serum at pH 5.5 and increases as the solution is made more alkaline. At pH 7.5 considerable turbidity results, although at this pH turbidities given by albumin and gamma globulin are negligible. In the latter instance it was shown that as small amounts of serum protein as 1.3 mg. per milliliter cause turbidity, whereas as much as 8 to 10 mg. of albumin or globulin are necessary to produce a corresponding amount of turbidity.

A consideration of these data suggests that there exists in human serum an acidic protein or other polymeric substance which reacts strongly with quaternary ammonium salts of the nature of those used in this work to form a precipitate. A mucoprotein of this type has been described by Winzler's group.[6] However, this protein, prepared from normal serum, was found to be unreactive with Octab in pH range of 6.0 to 10.0.

Similarly, the C-reactive protein found in acute phase sera might be responsible for causing turbidity when serum is treated with Octab. Adsorption of C-reactive protein from serum caused no decrease in the capacity of the serum to form turbidity with Octab.

The occurrence of a nondialyzable calcium-requiring bactericidal substance for *B. subtilis*[5] in acute phase serum suggested that this factor might by reacting with Octab. The inhibitory effect of phosphate buffer on development of turbidity also suggested a similarity between these two factors.

However, as has been stated, addition of *B. subtilis* to acute phase serum did not alter the turbidity reaction for Octab. Conversely, addition of increments of Octab to serum did not neutralize the bactericidal factors of serum for *B. subtilis.*

Whatever the characteristics of the turbidity-producing protein are, preliminary investigations suggest that it resides in the globulin fraction of human plasma.

SUMMARY

The reactivity of serum protein, human albumin, and gamma globulin with a quaternary ammonium salt, Octab, is described.

Under suitable experimental conditions normal and acute phase serum contains a component reactive with Octab which is neither albumin nor gamma globulin.

The nature of the reacting component is discussed.

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THE REACTION OF HUMAN SERUM WITH A QUATERNARY AMMONIUM SALT: RESULTS OF A SERIAL STUDY IN ACUTE RHEUMATIC FEVER

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IN A preceding communication[1] the reaction of whole serum and two components of serum, albumin and gamma globulin, with a quaternary ammonium salt was described. It was shown that the addition of octadecyl dimethyl benzyl ammonium chloride (Octab) to serum under controlled conditions of pH, salt concentration, and protein to detergent ratio caused turbidity formation which could not be due to either the albumin or gamma globulin fractions. The amount of turbidity was found to vary within a narrow range in a series of normal, healthy adults but increased significantly whenever disease was present. For example, patients with acute rheumatic fever are observed to have a high serum turbidity after addition of Octab, during the acute phase of the illness. During convalescence this reaction decreases and finally falls to normal turbidity ranges when a complete recovery from rheumatic fever has resulted. This report is a study of the serum-Octab turbidity reaction in a group of patients with rheumatic fever and rheumatoid arthritis.

EXPERIMENTAL METHOD

Patients selected for this study consisted of children admitted to the hospital with an unequivocal diagnosis of rheumatic fever. A prolonged follow-up study was possible since all of this group were discharged to a convalescent home for children where a careful clinical and laboratory observation was continued. In all instances but two, treatment consisted of salicylate therapy and bed rest during the acute stage of illness.

Blood samples were usually collected once a week during the acute phase of illness and once every two weeks during convalescence. These samples were obtained in sterile glass test tubes and the separated serum was immediately frozen and stored in rubber-stoppered glass tubes at −20° C. until use. Assay of serum for Octab turbidity was made according to a previously described method.[1] Sedimentation rate was determined by Wintrobe's method.[2] The bactericidal action of serum for *Bacillus subtilis* was assayed by a previously described technique.[3] The degree of inhibition of *B. subtilis* by the unknown serum is arbitrarily expressed as per cent inhibition. In each test, serum from a normal individual is inoculated at the same time as the unknown serum. The residual bacterial count in the normal serum after two hours of incubation at 37° C. is calculated as zero inhibition. The $\log_{10}$ of this number of bacteria is plotted on a graph against per cent inhibition. A line is drawn from zero inhibition (value of normal serum) to 100 per cent inhibition (no growth). Any intermediate inhibition by the unknown serum is then determined from this line by calculating the per cent inhibition from the $\log_{10}$ of the residual bacterial count in the unknown sera.

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RESULTS

Correlation of Serum-Octab Turbidity With Clinical and Other Laboratory Observations.—The observations shown in Figs. 1 to 7 were made on six different individuals with either acute rheumatic fever or chorea and on one patient with acute rheumatoid arthritis. Similar data have been obtained from an additional twelve patients with rheumatic fever and ten with rheumatoid arthritis. The results in this latter group were similar to those shown in Figs. 1 to 7.

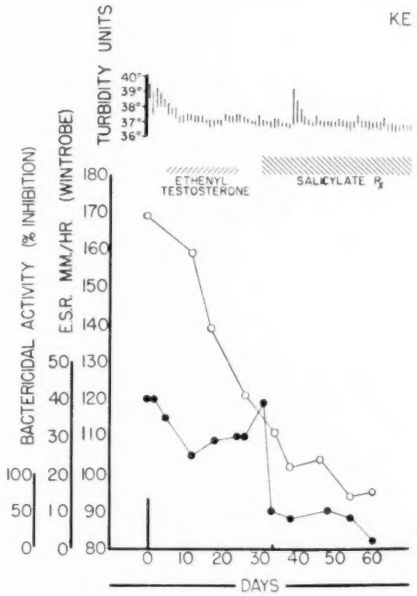

Fig. 1.—Patient K. E., with acute rheumatic fever, was given 300 mg. of ethenyl testosterone each day as indicated. Serum-Octab turbidity (open circles), erythrocyte sedimentation rates (solid circles), and bactericidal activity (bar graph) are shown. Temperature maxima and minima are illustrated in the upper part of the figure.

The first subject, Patient K. E. (Fig. 1), was a 13-year-old boy who was admitted to the hospital in January, 1950. At this time he was acutely ill with rheumatic fever and had migratory joint points associated with an effusion into the right knee joint. The initial laboratory study revealed an increased polymorphonuclear leucocyte count and an erythrocyte sedimentation rate (Wintrobe) of 40 mm. per hour. Blood serum obtained on the first day of hospitalization had an elevated Octab turbidity of 170 units and showed 70 per cent inhibition for *B. subtilis*. Three hundred milligrams of ethenyl testosterone were given on the second day and it was continued in this amount for twenty-three days. One week after admission the patient was afebrile and had no symptoms of active rheumatic fever. He remained asymptomatic and afebrile except for an episode of fever on the thirty-ninth day. This was attributable to an epidemic illness of diarrheal disease which similarly afflicted other children in the same hospital ward. It will be observed from Fig. 1 that serial assay of serum-Octab turbidity revealed a progressive fall of this

index during the first forty days of hospitalization. Correspondingly, a decrease of bactericidal activity of his serum took place, so that a negligible amount was present on the thirtieth day. In contrast to these findings were the values of the erythrocyte sedimentation rate. These persisted at high levels until salicylate therapy was started.

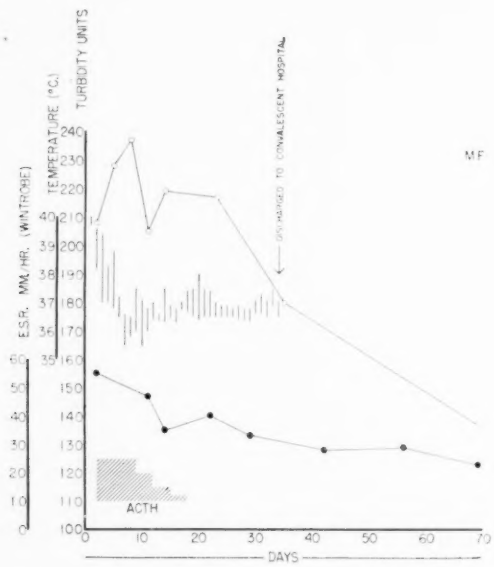

Fig. 2.—Patient M. F., with recurrent rheumatic fever, received 60 mg. of ACTH each day, reduced finally to 10 mg. a day as indicated. Maximum and minimum temperature changes are plotted with results of serum-Octab turbidity (open circles) and erythrocyte sedimentation rate (solid circles).

The assay of serum-Octab turbidity and bactericidal activity in this instance reflected accurately the clinical improvement of the patient. In contrast, erythrocyte sedimentation rate determinations remained elevated, despite absence of fever and tachycardia, and returned to normal only after salicylate therapy was begun.

In Fig. 2 observations made on Patient M. F., a 13-year-old boy admitted in July, 1950, with recurrent rheumatic fever, are shown. An initial attack of acute rheumatic fever was studied in August, 1948, at which time he was severely ill with myocardial and endocardial disease, complicated by erythema marginatum. During this period a persistent high elevation of serum-Octab turbidity was found, and this did not return to normal ranges until April, 1949. The recurrent attack shown in Fig. 2 was also severe and was associated with joint pains, fever, and signs and symptoms of heart failure. Adrenocorticotrophic hormone was administered in 15 mg. amounts every six hours on the second day and continued as shown in Fig. 2. On the sixth day the patient was afebrile, and all rheumatic symptoms had subsided. An initial serum-Octab turbidity index of 210 was found. Concomitant with administration of ACTH this value increased to 240 units and remained high

throughout the period of hormone therapy. Similarly there was little change in the erythrocyte sedimentation rate during this time. On the thirtieth day, however, the serum-Octab index decreased and fell to 138 units forty days later.

These data indicate that ACTH caused complete arrest of rheumatic symptoms but had little effect on rheumatic activity as reflected by the serum-Octab turbidity and erythrocyte sedimentation determinations. It is doubtful that ACTH actually stimulated an increase of serum-Octab turbidity since administration of this hormone to several normal individuals has not caused any change in the turbidity index.

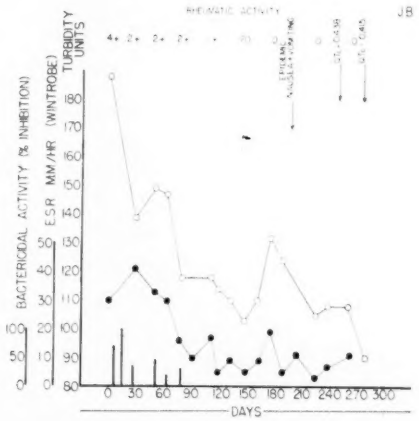

Fig. 3.—Patient J. B., with acute rheumatic fever. The severity of rheumatic activity is indicated at the top of the figure. Serum-Octab turbidity (open circles), erythrocyte sedimentation rate (solid circles), and bactericidal activity (bar graph) are shown during an observation period of nine months.

The course of Patient J. B., a 10-year-old girl with a primary attack of acute rheumatic fever, is shown in Fig. 3. It will be observed that persistent elevations of serum-Octab turbidity values, erythrocyte sedimentation rate, and bactericidal activity were found during the period of clinical evidence of rheumatic activity. It is of interest that a striking elevation of turbidity preceded by two weeks the onset of "epidemic" nausea and vomiting. This illness affected several other patients in the same hospital ward. Finally, after 270 days the serum-Octab turbidity index returned to normal levels. At this time all evidence of rheumatic activity was absent both by clinical and electrocardiographic observation.

Another example of the course of acute rheumatic fever is seen in Patient L. H., a 13-year-old girl whose laboratory data are shown in Fig. 4. An elevated serum-Octab turbidity index, an increased bactericidal effect for *B. subtilis*, and elevations of erythrocyte sedimentation rate were present during the acute phase of her illness. During convalescence, persisting unexplained fluctuations of serum-Octab turbidity index occurred. These could not be attributable to respiratory infection nor to any clinical evidence of rheumatic

activity. It is also of interest that in this patient the values of erythrocyte sedimentation rate persisted in the range of 20 mm. per hour. It is possible that these abnormalities represented evidence of clinically submerged rheumatic activity.

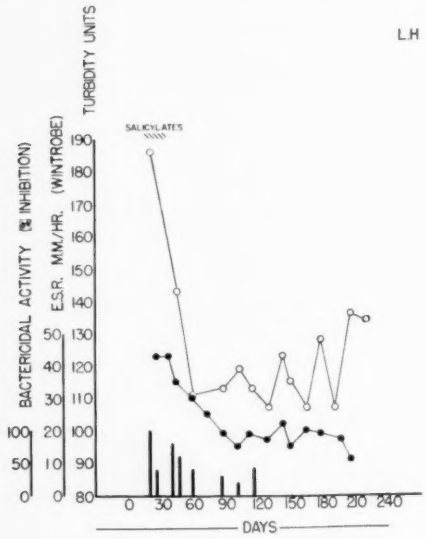

Fig. 4.

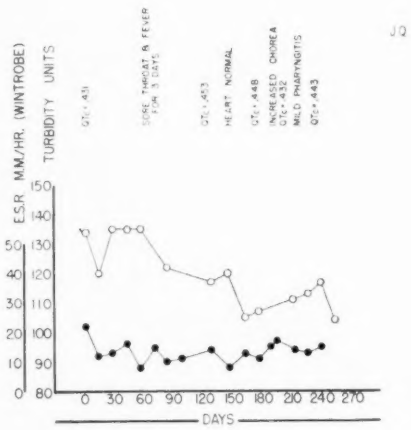

Fig. 5.

Fig. 4.—Patient L. H., with acute rheumatic fever. Serum-Octab turbidity is depicted by open circles, erythrocyte sedimentation rate by solid circles, and bactericidal activity by bar graph. No clinical evidence of rheumatic activity was present during the last three months of study.

Fig. 5.—Patient J. Q., with acute rheumatic chorea. Serum-Octab turbidity (open circles) is compared with erythrocyte sedimentation rate (solid circles). Observe that clinical activity of the chorea persisted throughout the period of study.

The results of study of a patient with acute rheumatic chorea (Patient J. Q.) are shown in Fig. 5. During a period of 250 days of observation this patient demonstrated clinical evidence of rheumatic activity. There was persistent chorea, an increase in QTc interval on ECG and intermittent respiratory infections. It is of interest, therefore, that although the erythrocyte sedimentation rates were always less than 20 mm. per hour, the serum-Octab turbidity values were persistently elevated. The observations of this and other patients reveal that an increased serum-Octab turbidity may be found even when an erythrocyte sedimentation rate of less than 20 mm. per hour is present.

In contrast to the observations of the preceding five individuals are the data obtained from Patient E. K. (Fig. 6), an 11-year-old boy who developed acute rheumatic fever in January, 1949. In the first week of his illness there was an increased erythrocyte sedimentation rate and bactericidal activity of the serum. A gratifying clinical response to salicylates was obtained, during which time a prompt decrease of erythrocyte sedimentation rate and bactericidal activity was noted. The first observation illustrated in Fig. 6 was made one month after the onset of illness. This patient remained under surveillance for 330 days, during which time he seemed to be completely recovered from rheumatic fever. During all this period, normal serum-Octab turbidity indices

as well as normal sedimentation rate values were obtained. Similar studies of less extended duration (120 to 200 days) have been made on six other patients who recovered from acute rheumatic fever.

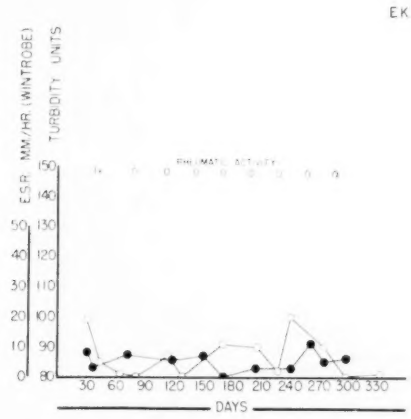

Fig. 6.—Patient E. K., with acute rheumatic fever, observed one month after onset of acute disease. During the eleven-month period of study no clinical evidence of rheumatic activity was present. Normal values of serum-Octab turbidity (open circles) and erythrocyte sedimentation rate (solid circles) were found during the entire period of observation.

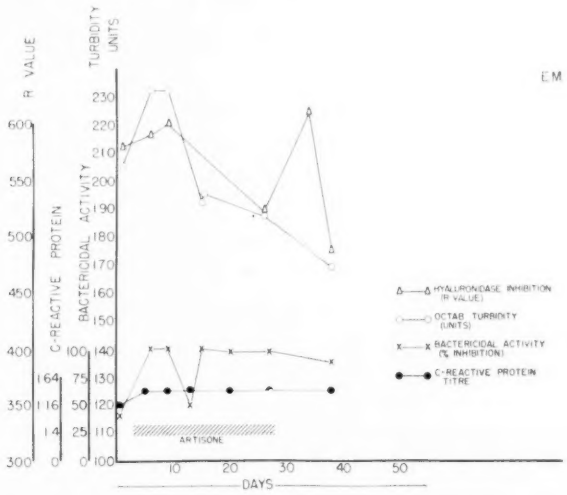

Fig. 7.—Patient E. M., with grade 2, stage 1 rheumatoid arthritis. This figure compares four nonspecific changes in blood serum resulting from the inflammatory reaction of arthritis. The abscissa represents the upper limit of normal for healthy adults for each test illustrated. Erythrocyte sedimentation rates (not shown) were persistently elevated throughout the observation period.

A comparison of serum-Octab turbidity with other nonspecific changes in human blood was made in a group of patients with rheumatoid arthritis. A representative example of these results is shown in Fig. 7 which illustrates observations made on a 49-year-old man (Patient E. M.) who had grade 2, stage 1 rheumatoid arthritis. He was given 300 mg. of artisone* each day during most of the study period with only slight clinical improvement.

*Artisone (Δ5-pregnene-3β, 21-diol-20 one, 21 monoacetate) obtained from Wyeth Incorporated, Philadelphia, Pa.

A battery of nonspecific tests for measurement of rheumatic activity was employed. Testicular hyaluronidase inhibition (done on fresh serum) was determined by Glick's method[4] and is expressed in terms of R value rather than per cent inhibition. The C-reactive protein titer was measured by addition of a standardized suspension of Formalinized Type 27 pneumococci to 0.5 ml. of serial twofold dilution of serum. The last tube to show definite capsular swelling is taken as the end point of the C-reactive protein.[5] Erythrocyte sedimentation values (not plotted in Fig. 7) were persistently elevated in a range of 38 to 40 mm. per hour.

An inspection of Fig. 7 reveals that a significant increase of all components was found. In each instance these values were persistently elevated with the possible exception of serum-Octab turbidity and testicular hyaluronidase inhibition levels. The slight clinical improvement of the patient was best reflected by the slight decrease of the values of these two assay techniques.

DISCUSSION

Many different changes in human blood and serum during acute infections or in acute rheumatic fever have been described. These include alteration of the erythrocyte sedimentation rate,[6] occurrence of an abnormal Weltmann serum coagulation,[7] presence of an abnormal C-reactive protein,[5] increase of testicular hyaluronidase inhibition,[8] increased bactericidal activity of serum for *B. subtilis*,[3] and other changes.

This description of the reaction of human serum with a quaternary ammonium salt (Octab) to form turbidity is an example of another type of protein change which occurs in acute phase sera. The mechanism by which these apparently unrelated stress manifestations are produced is not clear.

Regardless of the significance of these abnormalities in acute phase sera, it appears that measurement of turbidity after addition of Octab to serum may offer a simple and reliable assay of inflammatory reactions in human beings. In this investigation a serial study of serum-Octab turbidity in a group of eighteen patients with rheumatic fever and eleven individuals with rheumatoid arthritis indicates that this technique is a valuable laboratory aid in assessing rheumatic activity.

In view of the sensitivity of serum-Octab turbidity in detecting inflammatory reactions, an application of this study may provide some insight into the problem of submerged rheumatic activity in clinically "recovered" rheumatic fever patients. An increased serum-Octab turbidity found in rheumatic fever and rheumatoid arthritis is in no sense a specific measure of rheumatic inflammatory reaction. Similar increases are found in such unrelated pathologic conditions as pneumonia, poliomyelitis, carcinoma, and tuberculosis.

SUMMARY

Correlative studies of erythrocyte sedimentation rate, bactericidal activity of serum for *B. subtilis*, and serum-Octab turbidity have been described in a group of patients with rheumatic fever, chorea, and rheumatoid arthritis.

The serum-Octab turbidity index is shown to reflect accurately the degree of inflammatory activity in these diseases.

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ESTIMATION OF NONGLUCOSAMINE POLYSACCHARIDES OF THE SERUM PROTEINS WITH THE ANTHRONE REAGENT

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INTRODUCTION

Dreywood, in 1946, demonstrated that anthrone was a sensitive and specific reagent for the detection of carbohydrates.[1] A large number of carbohydrates, including mono-, di-, and polysaccharides, dextrins, bacterial polysaccharides, and cellulose derivatives yielded[1, 2] positive reactions, manifested by the development of a characteristic green color upon the addition of a solution of anthrone in concentrated sulfuric acid. The anthrone reaction was adapted for the quantitative estimation of certain carbohydrates,[2] including glycogen[3] and blood sugar.[4] Sattler and Zerban[5, 6] postulated that the anthrone reaction requires the initial formation of furfural or furfural derivatives from carbohydrates which give a positive reaction. The inability of desoxyribose, glucosamine, glucuronic acid, and certain other carbohydrates to produce the color seemed to be in conformity with an expected lack of furfural formation from these substances under the conditions of this reaction.

During studies on the serum glycoproteins of patients with cancer and other diseases, it was considered desirable to investigate the reaction of the anthrone reagent with certain protein-bound polysaccharide fractions of human serum.

MATERIALS AND METHODS

I. *The Determination of the Total Polysaccharides Bound to Serum Proteins.—Reagents:*

1. Absolute ethyl alcohol (99.9 per cent).
2. Anthrone reagent*—0.2 per cent anthrone (National Biochemical Company) in 96 per cent sulfuric acid.
3. 0.9 per cent saline solution.

Methods: The serum is diluted with twice the volume of 0.9 per cent saline. A 0.4 ml. aliquot of the diluted material, containing 0.134 ml. of serum, is pipetted dropwise into a 15 by 125 mm. Pyrex tube containing 10 ml. of absolute ethyl alcohol. The precipitate formed is thoroughly mixed and then centrifuged for ten minutes at 2,000 r.p.m. The supernatant is decanted and the precipitate is washed by suspension in 10 ml. of absolute ethanol, followed by centrifugation. Four milliliters of water are added to the alcohol-washed precipitate and the material is agitated until dissolved. To this solution 8 ml. of anthrone reagent are added slowly; the mixture is thoroughly stirred with a glass rod, and allowed to stand at room temperature for a period of fifteen minutes. At the end of this time, the solutions are transferred to colorimeter cuvettes and the optical density is measured

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*The anthrone reagent should be freshly prepared before use since it deteriorates on standing.

at a wave length of 620 mμ in a model 6A Coleman Junior spectrophotometer. A reagent blank made up of 4 ml. of distilled water and a set of standards (containing 0.2 and 0.1 mg. respectively of an equimolar mixture of galactose and mannose in 4 ml. of water) are treated in the same manner as the unknown solutions.

Calculations:

$$\frac{\text{O.D. unknown}}{\text{O.D. standard}} \times 0.2 \times \frac{1}{0.134} \times 100 = \text{mg. \% serum polysaccharide expressed as galactose-mannose.}$$

II. *The Determination of the Polysaccharides in the Muco-protein Fraction of Serum.—Reagents:*

1. Perchloric acid—6 per cent.
2. Phosphotungstic acid—5 per cent in 2N HCl.
3. Absolute ethyl alcohol (99.9 per cent).
4. Saturated solution of sodium carbonate (22 per cent).
5. Anthrone reagent.

Methods: To a measured volume of serum, preferably 3 to 5 ml., two volumes of 6 per cent perchloric acid are added. The precipitate formed is shaken and allowed to stand for exactly ten minutes, after which it is filtered through No. 50 Whatman filter paper. After complete filtration, a measured volume (2 to 3 ml.) of the filtrate, containing the serum mucoproteins, is pipetted into a 15 by 125 mm. Pyrex tube (the remainder of the filtrate may be used for the biuret protein determination) and 2 ml. of the acid phosphotungstate are added. A light flocculent precipitate is formed which is swirled gently and allowed to stand for fifteen minutes. At the end of this period, the tubes are centrifuged at 2,000 r.p.m. for ten minutes. The supernatant is decanted and the precipitate washed first with 5 ml. of the phosphotungstic acid and then with 10 ml. of absolute ethanol. The alcohol-washed precipitate is then dissolved in 0.5 ml. of saturated sodium carbonate and the solution is brought to a volume of 4 ml. with distilled water. Eight milliliters of the anthrone reagent are added with care* and the remainder of the procedure is carried out as described under section I.

Calculations:

$$\frac{\dfrac{\text{O.D. unknown}}{\text{O.D. standard}} \times 0.1}{\text{ml. serum} \times \dfrac{\text{ml. filtrate used}}{\text{ml. total filtrate}}} \times 100 = \text{mg. \% polysaccharide (expressed as galactose-mannose) in serum mucoprotein}$$

RESULTS AND DISCUSSION

The absorption spectral curves† derived from the reaction of anthrone with galactose, mannose, and an equimolar mixture of galactose and mannose were investigated, since it has been observed[7, 8, 9] that the polysaccharide of serum is composed of galactose, mannose, and glucosamine. The spectral curves shown in Fig. 1 demonstrate that the green-colored product obtained with equivalent amounts of galactose and mannose and with an equimolar mixture of these compounds were identical, showing absorption maxima at 620 mμ.

Dreywood[1] reported that proteins did not produce the specific green color when allowed to react with the anthrone reagent. Morris[2] noted that proteins

*A vigorous reaction with evolution of CO_2 takes place; therefore, extreme caution should be exercised on adding the anthrone reagent.

†The absorption spectrum data were determined on a model DU Beckman quartz spectrophotometer, using Corex cuvettes and a light path length equal to 1 centimeter.

form a red color with the anthrone reagent. Seifter and co-workers[3] reported that tryptophane alone, of the various amino acids tested, developed a red color which was specific enough to serve as a qualitative test for tryptophane in either the free or combined form. To ascertain whether the presence of proteins might interfere with the accurate estimation of the polysaccharide in serum glycoproteins, absorption spectral curves were determined on several protein fractions after reaction with anthrone.

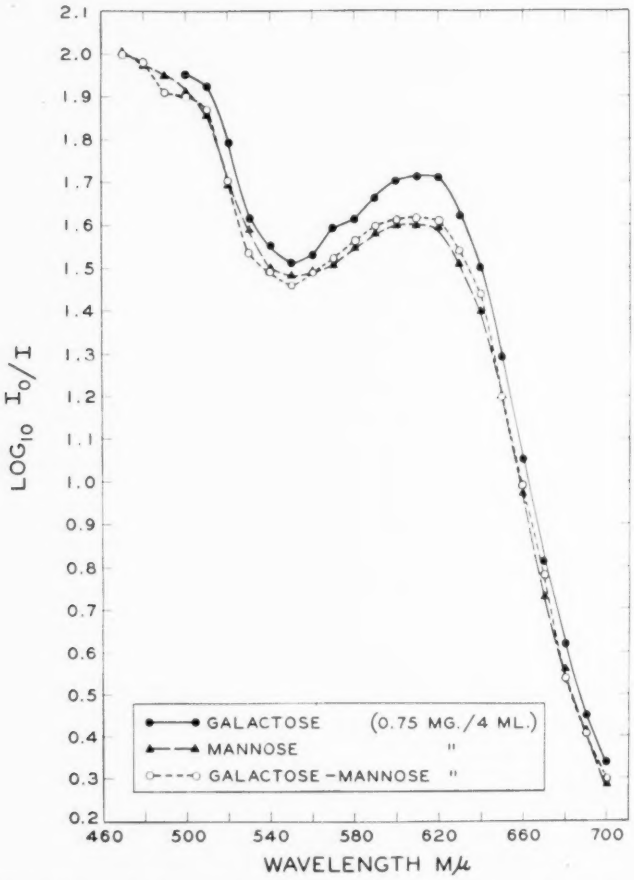

Fig. 1.—Comparison of absorption spectral curves of the products resulting from the reaction of galactose, mannose, and an equimolar mixture of galactose and mannose with the anthrone reagent.

Commercially available human serum albumin* when allowed to react with anthrone showed a maximum absorption at 520 mμ (Fig. 2). The absence of any appreciable amount of carbohydrate in this preparation of serum albumin was demonstrated by the lack of significant absorption at 620 mμ. A globulin sample of the same concentration as the albumin, obtained by Pillemer separation,[10] showed a maximum at 520 mμ as well as increased absorption at 620 mμ (Fig. 2). The increased absorption at 620 mμ appeared

*Obtained from the Cutter Laboratories, Berkeley, Calif.

to be related to the presence of carbohydrate material among the serum globulins. Since the preponderance of polysaccharide in the serum proteins has been observed to be in the alpha globulin fraction, the reaction of anthrone with a carbohydrate-rich component of alpha-1 globulin, i.e. the mucoprotein,[11, 12] isolated from a perchloric acid filtrate was investigated. The absorption spectral curve resulting from the reaction of anthrone with serum mucoprotein showed two peaks with absorption maxima at 620 mμ and at 520 mμ (Fig. 2).

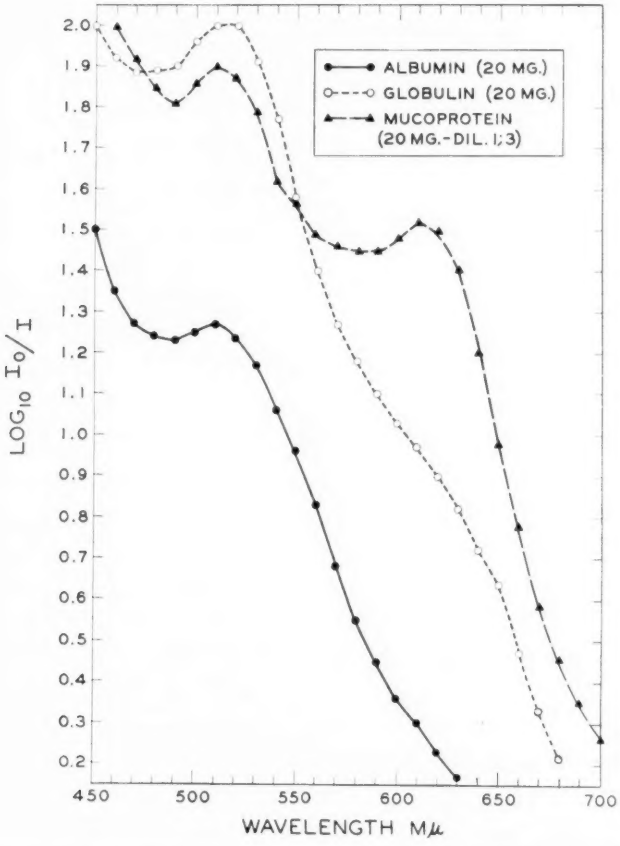

Fig. 2.—Comparison of absorption spectral curves of the products resulting from the reaction of albumin, globulin, and mucoprotein with the anthrone reagent.

The general shape of the curve and the maximum at 620 mμ agreed closely with that of an equimolar mixture of galactose and mannose (Figs. 1 and 2). In addition, the colored solution which developed with the mucoprotein complied with the Beer-Lambert law when examined at 620 mμ.

The colored product resulting from the anthrone reaction with an ethanolic precipitate of sera from normal and different pathologic conditions showed considerable variation in the ratio of absorption at 520 mμ and at 620 mμ (Fig. 3). Absorption at 620 mμ correlated with the polysaccharide content

(Table I). No quantitative correlation between either the nitrogen or the tryptophane content of the sera with the absorption at 520 mμ could be established. The products of the anthrone reaction with both the galactose-mannose mixure and the ethanol precipitated proteins of serum complied with the Beer-Lambert law at a wave length of 620 mμ.

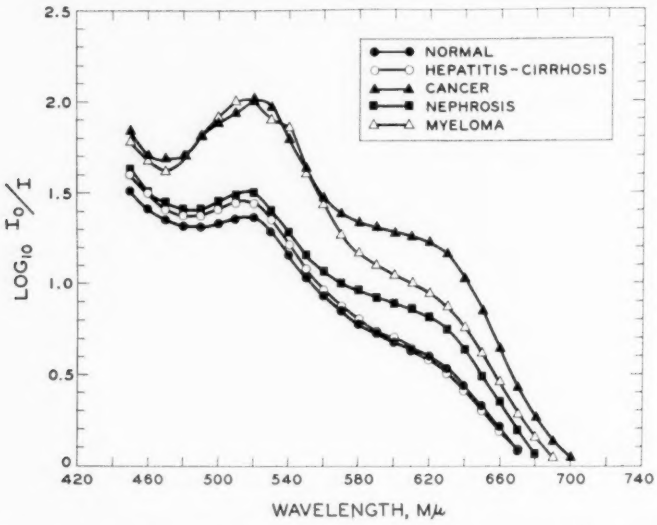

Fig. 3.—Comparison of absorption spectral curves of the products resulting from the reaction of ethanol precipitates of serum from normal and various pathologic conditions with the anthrone reagent.

Observations on the total serum proteins obtained by alcohol precipitation, after reaction with the anthrone reagent, were made at both 520 mμ and 620 mμ at specific intervals of time after addition of the reagent. This was also repeated with an equimolar mixture of galactose and mannose. The results shown in Fig. 4 demonstrate that the absorption at 520 mμ and that at 620 mμ reach maxima at the same time, i.e. approximately fifteen minutes. When equimolar mixtures of galactose and mannose were added to a representative protein, casein (100 mg. per milliliter of 1N NaOH), the intensity of the color developed at 620 mμ with anthrone was proportional to the concentration of added carbohydrate. On the basis of these observations, when

TABLE I. COMPARISON OF THE DETERMINATION OF SERUM PROTEIN POLYSACCHARIDE CONTENT BY THE ANTHRONE AND TRYPTOPHANE METHODS

METHOD	PROTEIN FRACTION	NUMBER OF SERA*	RANGE (MG. %)	MEAN VALUE (MG. %)	CORRELATION COEFFICIENT
Anthrone	Ethanol precipitate of serum	50	88.3-278	150.4	0.992
Tryptophane	Ethanol precipitate of serum	50	85.7-276	148.7	
Anthrone	Serum mucoprotein	15	8.8-63.2	24.6	0.995
Tryptophane	Serum mucoprotein	15	8.8-62.8	24.8	

*Normal and pathologic.

the optical density of the solution was measured at a wave length of 620 mμ at the time of maximum color development, the interference by color due to the reaction of the anthrone with the total serum proteins was negligible. Substances such as glucose, ascorbic acid, lactic acid, and uric acid which react with anthrone do not constitute interfering factors since these are present in the protein-free filtrate and are eliminated during the precipitation and subsequent washing with ethanol. Glucosamine does not react with anthrone and therefore does not constitute an interfering factor.

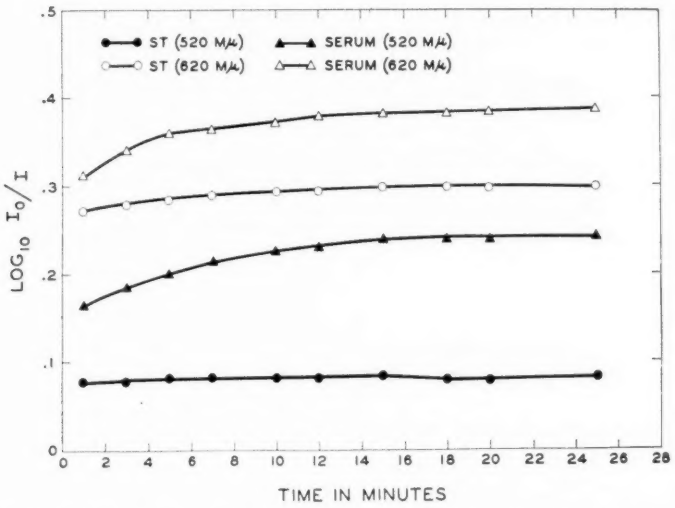

Fig. 4.—Rate of color development in the anthrone reaction with the galactose-mannose standard, and with ethanol precipitated protein of serum at 520 mμ and 620 mμ.

The polysaccharide values in the total serum proteins obtained from fifty samples of serum by this method were found to be in good agreement with those obtained by the tryptophane method of Shetlar and associates.[13] In addition, the results obtained by the anthrone method also showed a good correlation with those obtained by employing the tryptophane method in the estimation of the ratio, polysaccharide/biuret protein, of the serum mucoprotein (Table I).

CONCLUSION

A simple, rapid method for the estimation of the polysaccharide in serum protein fractions, utilizing the anthrone reaction with carbohydrates, is described. The method obviates the use of a carefully controlled condensation procedure. The results obtained by the anthrone reaction are in close agreement with other methods for the estimation of the serum polysaccharide content.

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THE TWENTY-FOUR HOURLY ENDOGENOUS CREATININE CLEARANCE AS A CLINICAL MEASURE OF THE FUNCTIONAL STATE OF THE KIDNEYS*

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FOR years there has been a continued effort to find a procedure for measuring the volume of the glomerular filtrate which possesses two characteristics: (1) that it is simple enough to be carried out in the clinic, and (2) that it is sufficiently accurate to permit reliable clinical evaluation of the functional state of the kidneys.

The first important attempts in this regard were made by Rehberg[1] who studied the excretion of intravenously administered creatinine, employing the principle that the urinary content of creatinine for a period of time, divided by the concentration of this substance in the plasma, would give the desired information. This necessitated the assumption that all of the creatinine reached the urine solely by glomerular filtration. Subsequently, Shannon,[2] Miller and Winkler,[3] and Jolliffe and Chasis[4] showed that exogenous creatinine, even at low plasma levels, is eliminated in part by tubular secretion. Accordingly, the exogenous creatinine clearance gives falsely high values for the volume of the glomerular filtrate. In 1935 Smith and Shannon[5] published substantial evidence that inulin, administered intravenously, is neither secreted nor reabsorbed by the tubules and hence its use gives a true value for the volume of the glomerular filtrate. While this inulin method has been, and continues to be, of fundamental importance in experimental work, it does not lend itself easily to clinical use because of major technical difficulties—the necessity for continuous intravenous administration, requiring sharp limitation of the physical activity of the patient—and because of restriction of observations to short periods of time.

These disadvantages have led to a search for a simpler and clinically more useful test. Presumably this goal could be reached by means of a substance which is produced as an end product of metabolism and which is neither reabsorbed nor secreted by the renal tubules. Miller and Winkler,[3] Steinitz and Turkänd,[6] and Brod and Sirota,[7] all, by performing simultaneous inulin and endogenous creatinine clearances, have shown that the volume of the glomerular filtrate as measured by endogenous creatinine clearance is the same as that determined by inulin clearance, and by inference, endogenous creatinine is neither reabsorbed nor secreted by the tubules in the case of normal human beings.

However, before the use of the endogenous creatinine clearance is recommended as a clinical test, the different sources of error require careful examina-

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tion. These are (1) technique for the determination of creatinine, (2) the creatinine content of food, since such creatinine is exogenous, and (3) the question of tubular secretion of endogenous creatinine in patients with renal disease.

I. Technique.—

It is agreed by all workers that the alkaline picrate method measures true creatinine in the urine. However, this does not hold for the serum or plasma.

Miller and Dubos[8] showed, by a specific enzymatic method, that the true creatinine content of plasma or serum is less than the amounts determined by the old Folin method. Subsequently, Miller and Winkler,[3] using this same specific method, obtained good agreement between the endogenous creatinine and inulin clearances in normal subjects. Bonsnes and Taussky's modification[9] of the Folin method, while more suitable for use in the modern colorimeters, continues to give values which are higher than those obtained with the specific enzymatic method. In our series, twenty-two determinations on the serum of eleven normal young men, using the Bonsnes and Taussky method, gave an average of 1.11 mg. per cent (range 0.95 to 1.29, see Table V). The average value obtained by Miller and Dubos, using their specific enzymatic method, was 0.99 mg. per cent, with a range of 0.62 to 1.24 mg. per cent. Since Miller and Dubos do not state at what time of the day the samples of blood were drawn, it is conceivable that the plasma creatinine values reported by them are higher in some instances than fasting values, since, as will be shown later, nonfasting plasma concentrations are increased by ingestion of the usual amounts of meat. This might also account for the wide spread of their serum creatinine values. For the normal women, we obtained an average of 0.86 mg. per cent (range 0.77 to 0.98 mg. per cent) by the Bonsnes and Taussky method.

A personal communication from Ephraim Shorr points out that Miss Hertha Taussky obtained lower values by the Bonsnes and Taussky method if a more concentrated filtrate is used. She attributes this to less accurate colorimeter readings with the greater dilution. However, on two occasions pure creatinine solution containing 1 mg. per cent gave the following results in our hands: dilutions 1:10 and 1:4 gave values of 1.05 and 0.98, and 1.03 and 0.98 mg. per cent respectively (Table I). But when these two dilutions are applied to the

TABLE I. CREATININE VALUES DETERMINED BY THE BONSNES AND TAUSSKY METHOD

SAMPLE OR SUBJECT	DATE (1950)	1:10 FILTRATE (MG. %)	1:4 FILTRATE (MG. %)	PER CENT DIFFERENCE
Creatinine standard 1 mg. %	6/23	1.05	1.03	1.9
Creatinine standard 1 mg. %	7/12	0.98	0.98	0
A. C. serum	6/9	1.26	0.99	27
B. A. serum	6/9	0.88	0.73	21
B. A. serum	6/12	0.80	0.66	21
H. S. serum	6/12	1.32	1.08	22
Plasma I (blood bank)	6/30	0.83	0.72	15
Plasma I (blood bank)	7/5	0.85	0.70	21
Plasma I (blood bank)	7/6	0.84	0.68	23
Plasma I (blood bank)	7/14	0.85	0.71	20

same samples of serum or plasma, we do get much lower values with the lesser dilution as shown in Table I. Further study indicates that the lower values obtained from the 1:4 filtrate are caused by adsorption of more creatinine on the denser protein precipitate. This is brought to light by eluting the two precipitates with water added in volumes proportional to the amount of precipitate. This resulted in the data shown in Table II-A. It is seen that the eluate from the 1:4 preparation has a concentration of creatinine roughly twice that from the 1:10 preparation. Further evidence in support of our contention that lower values are obtained with lesser dilutions due to adsorption of more creatinine on the denser precipitate is shown in Table II-B. It will be seen that the recovery of a known amount of creatinine added to the plasma before deproteinizing is definitely smaller when the serum is diluted 1:4 rather than 1:10. On the other hand, when known amounts of creatinine are added to the filtrates, the recovery is close to 100 per cent whether the dilution is 1:10 or 1:4.

TABLE IIA. RECOVERY OF CREATININE FROM PROTEIN PRECIPITATE OF PLASMA BY ELUTION WITH DISTILLED WATER

PREPARATION	I CREATININE CONTENT OF ORIGINAL FILTRATE (MG. %)	II CREATININE CONCENTRATION OF ELUATE (MG. %)
A 1:10 filtrate	0.848	0.038
1:4 filtrate	0.712	0.070
B 1:10 filtrate	0.752	0.030
1:4 filtrate	0.660	0.052

TABLE IIB. RECOVERY OF C. P. CREATININE ADDED TO DEPROTEINIZING MIXTURE AND TO CLEAR FILTRATE

	PER CENT RECOVERY
A Creatinine Added to Deproteinizing Mixture	
(1) 1:10 preparation	88
(2) 1:4 preparation	78
B Creatinine Added to Filtrate	
(1) 1:10 filtrate	96
(2) 1:4 filtrate	95

From these studies it would seem that when serum is deproteinized for the determination of creatinine, a small fraction of the latter becomes unavoidably adsorbed by the protein precipitate. The amount of creatinine lost through adsorption is less when a 1:10 filtrate is used than when a lesser dilution such as a 1:4 filtrate is employed. The small amount of creatinine lost in this way is compensated for by the presence of small amounts of noncreatinine chromogen in the plasma of normal individuals. Hence it is through this fortunate combination of errors that a close correlation between the inulin and endogenous creatinine clearances is obtained.

II. *Effect of Preformed Creatinine in Meat on Creatinine Clearance.*—

As long ago as 1933 Ziekelbein[10] reported that ingestion of large amounts of cooked meat was accompanied by a significant increase in his own excretion of creatinine. Shannon[2] in 1935 showed that when creatinine is administered, some of it is eliminated by tubular secretion.

Presumably the organism rids itself of some of the preformed creatinine present in meat also by tubular secretion. Since uncooked muscle contains much creatine and little creatinine, we subjected beef to different methods of cooking and determined the increase in the creatinine content. Table III gives the data on these studies. It also gives the creatinine content of boiled ham and baked lamb and perch. It is seen that the creatinine content of the meat increases with higher cooking temperatures.

TABLE III. CREATINE AND CREATININE CONTENT OF DIFFERENT MEATS WITH DIFFERENT METHODS OF PREPARATION

MEAT	METHOD OF COOKING*	CREATININE CONTENT (MG. PER GM. WET WEIGHT)	CREATINE CONTENT (MG. PER GM. WET WEIGHT)
Ground round steak	Uncooked	0.42	4.90
Ground round steak	Baked in dry heat	3.20	0.40
Ground round steak	Baked in moist heat	2.00	
Ground round steak	Boiled in water	1.54	
Ham (boiled)		0.77	
Lamb	Baked in moist heat	1.71	
Perch	Baked in moist heat	0.75	

*Cooking time, 45 minutes.

Next, the serum concentration of creatinine and the creatinine in the urine were determined in subjects fed diets containing various amounts of meat as compared to values obtained while on a meat-free diet. One of the subjects, while on a meat-free diet, was also given chemically pure creatinine in amounts equal to those calculated to be present in 450 grams of beef baked in moist heat. The effect of the intake of various amounts of exogenous creatinine upon the serum concentration and the clearances are shown in Table IV. The data show that meat in the diet definitely affects the value of the 24-hourly creatinine clearance. Subject W. B. on a 40 Gm. protein meat-free diet gave a clearance of 156.5 liters; when the protein content of the diet, still meat-free, was increased to 120 Gm., the clearance was 157.0 liters. On a 75 Gm. protein diet with meat, the clearance rose to 168 liters, and on a 120 Gm. protein meat diet, the clearance was as high as 218 liters. Comparable results were obtained in Subject B. R. Obviously, the protein content of the diet, as long as it is not derived from meat, does not affect the creatinine clearance. On the other hand, meat in the diet, on account of its creatinine content, gives falsely high values for the volume of the glomerular filtrate.

It is seen that the level of fasting serum creatinine bears no relation to the different diets. However, following a meal containing meat, there is a transient but significant rise in the concentration of serum creatinine. Serum specimens drawn at noon before ingestion of meat gave essentially the same values for concentration of creatinine as those obtained in the fasting state. In Subject W. B. administration of chemically pure creatinine equal in amounts to that calculated to be present in the high meat diet resulted in elevation of serum creatinine concentration to the same extent as that observed after ingestion of the meat. On another occasion, repeated hourly administration of smaller doses of creatinine resulted in a sustained elevation of the concentration of serum creatinine.

TABLE IV. FASTING AND HOURLY SERUM CREATININE CONCENTRATIONS IN RELATION TO VARIOUS DIETS, TO ADMINISTRATION OF CREATININE AND CARONAMIDE, AND THE CORRESPONDING CLEARANCES (VALUES OBTAINED ON 4TH AND 5TH DAYS OF DIET)

SUBJECT	DIET	SERUM CREATININE (MG. %)						CREATININE CLEARANCE L/24 HR.
		FASTING	NOON PRE-LUNCH	POST-PRANDIAL 1 HR.	2 HR.	3 HR.	4 HR.	
W. B.	40 Gm. protein, meat-free	1.27 1.18						156.5
	40 Gm. protein, with 75 Gm. beef	1.30 1.16	1.32	1.28	1.24	1.24	1.28	163.0
	75 Gm. protein, with 75 Gm. beef and 60 Gm. cooked ham	1.20 1.26	1.16	1.32	1.40	1.24	1.40	168.0
	120 Gm. protein, meat-free	1.12 1.16						157.0
	120 Gm. protein, with 450 Gm. beef	1.14 1.06 1.20	1.08	1.39	1.47	1.37	1.36	218.0
	Same diet as above, but with caronamide administration 4.0 Gm. every 4 hr. starting on 3rd day of diet: After 36 hr. of caronamide After 60 hr. of caronamide	 1.58 1.97	 1.68	 2.01	 2.04	 2.04	 1.99	 159.0
	75 Gm. protein, meat-free, with C. P. creatinine—450 mg. with noon meal and same amount with evening meal on 4th experimental day	1.03 1.03	1.02	1.47	1.42	1.37	1.22	173.5
	Same diet as above, with creatinine administration—60 mg. every hour from 8:00 A.M. to 5:00 P.M. on 4th day	1.03 1.17 1.12	1.47	1.42	1.46	1.47	1.47	190.0
B. R.	40 Gm. protein, meat-free	1.18 1.13	1.16	1.19	1.22	1.19	1.19	146.5 147.3
	75 Gm. protein, with 75 Gm. beef	1.15 1.07	1.20	1.36	1.39	1.37	1.37	145.6 145.5
	120 Gm. protein, with 450 Gm. beef	1.15 1.08	1.04	1.31	1.36	1.32	1.33	181.7 191.1
	Same diet as above	1.20 1.13						
	Same diet as above, with caronamide 4 Gm. every 4 hr. starting on 3rd day of diet: After 36 hr. of caronamide After 60 hr. of caronamide	 1.57 1.57	 1.57	 1.75	 1.85	 1.83	 1.79	 142.7 147.9

When the subjects were on the high meat diets (See Table IV), administration of caronamide* caused a further increase in the concentration of serum creatinine. The creatinine excretion was elevated also, but to a lesser extent than on the same diet without caronamide. The net result of caronamide administration was a drop in the creatinine clearance while on a high meat diet to a level comparable with values obtained while on the meat-free diet. Since Jolliffe and Chasis[4] have shown that exogenous creatinine in man is partly secreted by the renal tubules even at low plasmal levels of 1.8 and 2.4 mg. per

*Caronamide used in this study was generously supplied by Sharp & Dohme, Inc., Philadelphia, Pa.

cent while endogenous creatinine is not excreted by this mechanism at similar low levels, and since caronamide is known to block the tubular secretion of a number of substances[11] and has been shown recently not to affect significantly the glomerular filtration rate,[12] these data afford presumptive evidence that some of the ingested creatinine (exogenous) is eliminated by tubular secretion.

Data obtained when subjects were transferred from a high-meat to a meat-free diet showed that the effect of the earlier diet on the urinary creatinine had disappeared after forty-eight hours. In addition, after this preparation, the concentration of creatinine in the serum does not vary significantly during the 24-hour period.

Furthermore, after a diet containing only 40 Gm. of protein derived in part from meat, the urinary creatinine is usually not significantly greater than the amount excreted on a meat-free diet. Accordingly, in order to obtain an entirely satisfactory value for the volume of the glomerular filtrate, the subject must have been restricted to a low protein diet containing not more than 75 Gm. of meat (raw weight) for at least two days. This is justified by our studies which indicate that beef which has been baked in moist heat contains about 2 mg. of creatinine per gram of raw weight, and that following ingestion, less than 50 per cent of this creatinine subsequently appears in the urine. Accordingly since a 40 Gm. protein diet would contain at most 75 grams of meat, the amount of exogenous creatinine excreted in the 24-hourly urine would not be more than 75 milligrams. This amount is approximately only 5 per cent of the usual endogenous creatinine excretion, and this degree of variation in the urinary content of creatinine occurs even on a constant meat-free diet.

III. *The Tubular Secretion of Endogenous Creatinine in Renal Disease.*—Miller and Winkler[3] and Brod and Sirota[7] have shown that in some examples of renal disease the volume of the glomerular filtrate as measured by the clearance of endogenous creatinine is greater than that determined by the inulin clearance. Miller and Winkler obtained a creatinine to inulin clearance ratio averaging 1.38 in such patients. Since the specific enzymatic method employed by them determines only true creatinine and since it is known that the non-creatinine chromogen increases in the serum of nephritic persons,[8] they would obtain lower values for serum creatinine in such patients than those determined by the alkaline picrate method. Furthermore, since it is believed that some of the endogenous creatinine is secreted by the renal tubules in the advanced nephritic condition, this combination of circumstances would account for the high creatinine clearances obtained by Miller and Winkler when compared with inulin clearance.

On the other hand, Brod and Sirota,[7] using the Bonsnes and Taussky method[9] for determining creatinine, studied thirteen patients with renal disease. They report an average creatinine to inulin clearance ratio, excluding data on one subject, of 1.04 ± 0.109 (range 0.89 to 1.25). How is this good agreement between inulin and creatinine clearance in subjects with renal disease to be explained? It has already been pointed out that the Bonsnes and Taussky method determines total chromogen which includes a greater proportion of non-

creatinine chromogen in the serum of advanced nephritic patients than in the case of normal subjects. It has also been stated that some endogenous creatinine is secreted by the renal tubules in such subjects. This gives an increase in the values of "creatinine" in both serum and urine, and evidently to a somewhat proportional extent. This fortuitous circumstance results in endogenous creatinine clearances which are only slightly higher than simultaneous inulin clearances in nephritic patients. As Brod and Sirota state, "A discrepancy of 10 per cent or greater generally appears only in subjects with filtrate rates below 40 c.c. per minute . . ." (which is equivalent to 58 liters per twenty-four hours, roughly a clearance reduced to one-third of normal), and that ". . . in such subjects, the absolute difference in the clearance values are of such small magnitude as to leave the endogenous chromogen clearance a clinically useful test."

IV. *Clinical Evaluation of the 24-Hourly Endogenous Creatinine Clearance Test.*—

A number of workers have shown that the volume of the glomerular filtrate varies throughout the twenty-four hours, because of physical activity,[13] drugs,[14] emotional tension,[15] and variations in intra-abdominal[16] and venous pressure.[17] Accordingly, the most realistic test is one that includes the whole 24-hour cycle. The inulin procedure is too disturbing to the patient and technically too difficult to be employed routinely for this purpose.

We have carried out simultaneous 24-hour clearances of urea and endogenous creatinine. On some patients, we also performed the hourly urea clearance test on the same day that 24-hour clearances were done, in order to compare the endogenous creatinine clearance with a test that is widely accepted as a measure of renal function.

Since the hourly urea test is subject to serious error because of inaccurate timing, all of these latter tests were performed while the patients were lying or sitting quietly in our laboratory, and they were made to void at exactly the end of the first and second hours.

Most of the 24-hour urine collections were done under personal supervision of the authors in the medical wards and in the Metabolism Unit. A few were trusted to outpatients who were given written instructions, and, judging by the relative constancy of the urinary creatinine values, we were satisfied that the collection had been done carefully. Occasionally an unusually low or high urinary creatinine value suggested faulty collection and such specimens were discarded.

Young, healthy, adult laboratory workers and medical students served as control subjects, all of whom were placed on a meat-free diet for two days preceding and during the test. They went about their usual activities during the period of collection. Thirty-two 24-hourly clearance studies were done on sixteen adults, five women and eleven men. Seventy-four clearance studies were performed on twenty-two patients with varying degrees of impairment of renal function. All of these patients were on a 40 Gm. protein diet. Nineteen of them had a clinical diagnosis of glomerulonephritis in all its different stages ranging from acute to terminal; on four of them in the latter stage (H. R., F. W., D. P., and M. K.), studies were done up to just a few days prior to death. In addition, the group includes one patient (D. M.) with polycystic kidneys, one (E. L.) with an endeterminate diagnosis of potassium deficiency of unknown etiology, and C. H., and B. C., both of whom carry a diagnosis of "essential" hypertension.

On those days when the standard hourly urea clearance test was performed on patients on the same day as a 24-hour collection was being obtained, values for urea nitrogen and creatinine in the hourly samples were added to those of the rest of the urine specimen, thus completing values for the 24-hour cycle.

TABLE V. 24-HOURLY ENDOGENOUS CREATININE CLEARANCE OF NORMAL SUBJECTS ON MEAT-FREE DIET*

SUBJECT	SERUM CREATININE (MG. %)	CREATININE CLEARANCE		
		ACTUAL (L./24 HR.)	CORRECTED TO 1.73 SQ.M. (L./24 HR.)	PER CENT OF AVERAGE
Men				
A. C., 30 Ht. 5′9½″ Wt. 160 lbs. S.A. 1.87	1.10 1.15	125.0 127.5	115.6 117.9	84 86
L. L., 40 Ht. 5′7″ Wt. 140 lb. S.A. 1.72	1.15 1.15	129.4 125.2	130.2 126.0	95 92
M. R., 27 Ht. 5′9½″ Wt. 160 lb. S.A. 1.87	1.02 0.99	168.2 161.6	155.6 149.5	113 109
H. S., 31 Ht. 6′0″ Wt. (ideal) 180 S.A. (ideal) 2.02	1.29 1.26	158.6 162.3	135.8 139.0	99 101
M. B., 23 Ht. 5′8″ Wt. 145 lb. S.A. 1.77	1.08 1.02	142.5 145.2	139.3 141.9	101 103
T. B., 21 Ht. 6′0″ Wt. 180 lb. S.A. 2.02	1.14 1.05	150.0 169.5	128.5 145.2	94 106
W. B., 28 Ht. 6′1½″ Wt. 178 lb. S.A. 2.02	1.15 1.17	150.2 163.9	128.6 140.4	94 102
B. R., 29 Ht. 5′8″ Wt. 156 lb. S.A. 1.83	1.16 1.13	146.5 147.3	138.5 139.3	101 101
E. W., 23 Ht. 5′10¾″ Wt. 148.5 lb. S.A. 1.84	1.04 1.06	146.3 137.3	137.6 129.1	100 94
S. F., 32 Ht. 5′10½″ Wt. 165 lb. S.A. 1.92	0.95 0.98	171.8 161.9	154.8 145.9	113 106
J. J., 26 Ht. 5′9½″ Wt. (ideal) 160 lb. S.A. (ideal) 1.87	1.15 1.10	150.7 155.9	139.4 144.2	101 105
Averages	1.11	149.9	137.4 (range 116-156)	
Women				
M. M., 24 Ht. 5′6½″ Wt. 128 lb. S.A. 1.64	0.80 0.77	134.8 139.9	142.2 147.6	104 108
A. G., 24 Ht. 5′2½″ Wt. 116 lb. S. A. 1.52	0.83 0.88	119.3 121.0	135.8 137.7	99 101

(Continued on opposite page.)

TABLE V—CONT'D

SUBJECT	SERUM CREATININE (MG. %)	CREATININE CLEARANCE		
		ACTUAL (L./24 HR.)	CORRECTED TO 1.73 SQ.M. (L./24 HR.)	PER CENT OF AVERAGE
B. B., 24 Ht. 5'6" Wt. 125 lb. S. A. 1.63	0.98 0.94	106.9 113.1	113.4 120.0	83 88
B. R., 25 Ht. 5'4" Wt. 118 lb. S.A. 1.55	0.84 0.84	125.2 116.1	139.7 129.6	102 95
Y. D., 23 Ht. 5'5" Wt. 120 lb. S.A. 1.58	0.84 0.90	144.4 133.1	158.1 145.7	115 106
Averages	0.86	125.4	137.0 (range 113-158)	

*These clearances were obtained on the third and fourth days of meat-free feeding.

All urine specimens were preserved with toluene and were analyzed at the conclusion of each period.

The method of Gentzkow[18] was used to determine urea nitrogen in the blood. Urinary urea nitrogen was analyzed using Barker's method.[19] The determination of serum and urinary creatinine is discussed in section I.

The 24-hour clearances were calculated by simply dividing the 24-hour urinary excretion, expressed in milligrams, by the concentration in the blood (for urea) or serum (for creatinine), as the case may be, expressed in milligrams per liter.

The actual 24-hour values for endogenous creatinine clearances were converted to per cent of normal as follows (normal having been determined as 137 liters per 1.73 sq.M. body surface per 24 hours):

$$\frac{1.73}{\text{Actual body surface}} \times \text{Actual clearance} = \text{Corrected clearance}$$

$$\frac{\text{Corrected clearance}}{137} \times 100 = \text{Per cent of normal}$$

RESULTS

Table V presents the clearance data from the control subjects on a meat-free diet. First consideration will be given to the creatinine clearances.

Thirty-two clearances on sixteen normal subjects gave an over-all average of 137 liters per twenty-four hours, the range being 113 to 158 liters per 1.73 sq.M. of body surface.

There are seventy-four determinations of endogenous creatinine clearance on twenty-two patients (Table VI). They include two patients, R. C. and R. G., who were observed first in the acute stage with gross hematuria. At the other extreme are five terminal cases, F. W., D. M., D. P., M. K., and D. B.

Table VI also presents the data on simultaneous 24-hourly urea clearances, and hourly urea clearances from ten of the subjects with renal disease.

In order to reduce the creatinine clearance values to figures comparable with an established renal function test such as the hourly urea clearance, they have been converted to per cent of normal for the individual's actual body surface

TABLE VI. SIMULTANEOUS 24-HOURLY ENDOGENOUS CREATININE AND UREA CLEARANCES AND SOME HOURLY UREA CLEARANCES ON PATIENTS WITH RENAL DISEASE

SUBJECT	DATE	SERUM CREATININE (MG.%)	B.U.N. (MG. %)	24-HOURLY CREATININE CLEARANCE L./24 HR./1.73 SQ.M.	24-HOURLY CREATININE CLEARANCE % OF NORMAL	24-HOURLY UREA CLEARANCE (% OF NORMAL*)	HOURLY UREA CLEARANCE PERIOD I (% OF NORMAL)	HOURLY UREA CLEARANCE PERIOD II (% OF NORMAL)
J. P.	1/25/49	0.90	8.9	120.0	88	80	70	67
F, 29	3/25/49	0.86	10.1	121.8	89	82		
S.A. 1.76 sq.M.	6/14/49	0.94	12.0	120.6	88	66		
	9/28/49	0.84	9.8	117.4	86	71		
C. S.	4/5/49	1.55	14.7	107.2	78	60		
M, 33	5/11/49	1.77	13.3	92.2	67	56		
S.A. 1.95 sq.M.	6/22/49		11.4				67	60
	7/20/49	1.66		98.3	71			
	1/16/50	1.64	15.5	98.2	71	74		
	7/15/50	1.65		106.9	78			
	10/14/50	1.63		107.0	77			
J. W.	1/13/49	0.95	9.7	111.0	81	74		
F, 18								
S.A. 1.50 sq.M.								
E. L.	5/28/49	1.15	9.8	86.5	63	53		
F, 51								
S.A. 1.82 sq.M.								
M. W.	2/28/49	1.66	16.9	79.0	58	46	46	53
M, 21	10/11/49	2.25	20.9	67.9	50	39		
S.A. 1.71 sq.M.								
J. L.	2/8/49	1.15	10.8	66.6	49	43		
F, 29	2/17/49	0.96	6.7	72.1	53	42		
S.A. 1.83 sq.M.	2/28/49	1.03	7.3	72.3	53	44		
C. W.	6/28/49	1.44	15.9	79.1	58	48		
F, 30								
S.A. 1.62 sq.M.								
B. W.	1/18/49	2.18	22.4	67.9	49	40		
M, 15	1/21/49	2.20	23.0	62.9	46	38	34	34
S.A. 1.69 sq.M.								
W. C.	6/30/49	1.95	40.7	62.2	45	15		
M, 14								
S.A. 1.67 sq.M.								
R. L.	3/22/49	3.08	23.2	52.5	38	25		

*Urea clearance as liters per twenty-four hours reduced to cubic centimeters per minute and calculated as per cent of average values of 75 c.c. or 54 c.c. depending on urine volume.

R. L.	3/22/49	3.08	23.2	52.5	38	25		
M, 28	3/23/49	3.12	22.4	53.2	38	25	32	30
S.A. 1.72 sq.M.	3/30/50	5.82	46.0	21.6	16	17		
	6/12/50	7.92		19.0	14			
D. B.	11/24/48	1.55	14.8	57.8	42	27		
F, 16	1/27/49	2.18	18.8				28	21
S.A. 1.52 sq.M.	4/14/49	2.90	50.7	32.1	23	14		
	5/19/49		45.6				20	23
	12/22/49	3.76		34.4	25	11		
	2/27/50	5.45	44.6	16.2	12	10		
	6/10/50	6.38	51.9	12.5	9.1	7.4		
	9/29/50	11.20	117.3	7.3	5.3	5.9		
	10/30/50	15.50	137.7	4.3	3.1	2.5		
C. H.	10/9/48		29.3				38	39
M, 45	10/20/48	2.90		48.0	35			
Ideal S.A. 1.70 sq.M.	10/22/48		26.7				39	38
	12/2/48	3.92	27.6	35.6	26	27	24	23
B. C.	10/12/49	3.78	39.9	35.6	26	27		
M, 51	11/21/49	3.98	28.9	35.9	26	36		
S.A. 1.89 sq.M.	12/19/49	4.26	29.3	27.0	20	28		
R. C.	3/9/49	3.78	36.7	37.5	27	24	23	24
M, 42	3/16/49	3.68	47.7	38.7	28	20	28	25
S.A. 1.79 sq.M.	5/13/49	1.65	8.1	87.0	64	57	79	59
	7/25/49	1.52	10.1	101.0	74	80		
	12/12/49	1.28	11.6	113.5	83	64		
	6/5/50	1.15	10.8	137.6	100	92		
R. G.	6/22/49	6.40	51.0	24.8	18	19	16	17
M, 35	7/18/49	3.24	36.3	41.3	30	24		
S.A. 2.07 sq.M.	11/7/49	1.40	15.0	116.6	85	76		
E. C.	3/17/49	6.36	53.4	18.6	14	14		
M, 41								
S.A. 1.88 sq.M.								
F. W.	11/29/48	7.08	64.3	15.1	11	9	10	9.2
M, 26	11/30/48	7.15	62.1	14.9	11	9	8.7	11.5
S.A. 1.87 sq.M.	12/1/48	7.00	60.4	15.1	11	10	11.6	10.5
	2/18/49	12.60	137.5	3.7	2.6	3.5	5.3	4.6
	3/16/49	11.70	141.4	7.0	5.0	6.0		
	3/28/49		106.8				6.3	6.4
	4/1/49	9.75	118.4	5.8	4.2	4.8		
	5/28/49	12.20	119.8	4.6	3.3	3.4		
	7/21/49	15.30	139.9	3.5	2.5	3.4		
	9/8/49	17.70	181.4	2.0	1.5	1.9		
	9/13/49	18.90	219.8	0.4	0.3	0.3		
	9/19/49	24.00	263.3					

(Concluded on following page.)

TABLE VI—CONT'D

SUBJECT	DATE	SERUM CREATININE (MG. %)	B.U.N. (MG. %)	24-HOURLY CREATININE CLEARANCE		24-HOURLY UREA CLEARANCE	HOURLY UREA CLEARANCE	
				L./24 HR./1.73 SQ.M.	% OF NORMAL	(% OF NORMAL*)	PERIOD I (% OF NORMAL)	PERIOD II (% OF NORMAL)
B. K. M, 21 S.A. 1.92 sq.M.	9/8/49	9.53	52.6	11.9	8.7	8.3		
D. P. F, 37 S.A. 1.41 sq.M.	6/10/49	8.75	72.4	9.8	7.2	7.5		
	9/8/49	10.97	93.0	9.4	6.9	7.3		
	10/3/49	18.40	206.0	4.0	2.9	2.4		
D. M. M, 49 S.A. 1.71 sq.M.	2/10/49	13.40	118.2	9.7	7.1	8.8		
	4/28/50	12.80	116.3	4.9	3.6	5.3		
	5/5/50	13.20	109.2	4.1	3.0	4.2		
	5/17/50	14.80		3.2	2.3			
	5/20/50	16.60		2.8	2.0			
	5/24/50	16.80		0.9	0.7			
M. K. F, 65 S.A. ideal 1.55 sq.M.	7/12/49	11.20	99.0	6.6	4.8	6.1		
	8/10/49	13.20	74.7	7.1	5.2	7.4		
	4/5/50	12.80	163.7	5.5	4.0	5.3		
	7/19/50	15.50	141.2	4.6	3.4	4.2		
	10/16/50	15.80	131.7	3.3	2.4	2.8		
	11/1/50	19.10	158.1	2.0	1.5			
H. R. M, 44 S.A. 1.69 sq.M.	1/5/49	17.8	83.4	3.9	2.8	7.1		
	1/8/49	20.2	152.5	3.2	2.3	3.7		

area. For C. H., who was grossly obese, the ideal weight and body surface for his height were used. Table VI shows that when meticulous care is taken to obtain precise timing and when the analyses are carried out accurately, the 24-hourly creatinine and urea clearances, and also the hourly urea clearance, all expressed as per cent of normal, usually give comparable values.

However, the hourly urea clearance tests did not always prove satisfactory in the sense that good checks were not obtained between the two hourly values even with all the collections supervised closely and all the chemical determinations done by us. In one study on patient R. C. the first period gave 79.3 per cent, whereas the second period was 58.6 per cent; these figures would have left us in doubt regarding the status of renal function at that time had it not been for a simultaneous 24-hour creatinine clearance (64 per cent of normal) showing definite impairment of renal function.

The clinical data on these patients are given in Table VII.

DISCUSSION

Sirota, Baldwin, and Villarreal,[20] by a special device, injected inulin intravenously over 24-hour periods into subjects without kidney disease. They compared the endogenous creatinine clearance with the inulin clearance and obtained an average creatinine to inulin ratio of 0.95 ± 0.10. The average volume of the glomerular filtrate by the creatinine clearance was 106.2 ml. per minute, or 153 liters per twenty-four hours, corrected to body surface of 1.73 sq.M. These authors used the original Bonsnes and Taussky method for determining creatinine in the urine and plasma, which employs a 1:10 dilution of the serum or plasma.

Somewhat earlier, Brod and Sirota[7] made comparisons between the inulin and endogenous creatinine clearances on normal subjects for periods of only ten to twenty minutes. They also employed the Bonsnes and Taussky procedure, but used 1:4 filtrates of the plasma. The average ratio of creatinine to inulin clearance in their series was 1.00 ± 0.018 (range 0.88 to 1.10). The volume of the glomerular filtrate determined by the creatinine clearance was 120.8 ml. per minute, or 174 liters per twenty-four hours, corrected to body surface of 1.73 sq.M. Since they used 1:4 filtrates of the plasma, their creatinine values may be assumed to be smaller than those obtained by Sirota and co-workers[20] who used the 1:10 dilution, and this would account for the larger filtrate volumes reported by Brod and Sirota. Furthermore, the filtrate volumes measured over a continuous 24-hour period are more reliable than those calculated for an interval of a few minutes. In this laboratory, thirty-two endogenous creatinine clearances on sixteen normal subjects, using the Bonsnes and Taussky method with 1:10 dilutions of the serum, gave an average value of 137 liters per twenty-four hours, corrected to body surface of 1.73 sq.M. Since the subjects used by Sirota and co-workers[20] were recumbent and receiving continuous infusion of inulin solution throughout the twenty-four hours, their serum creatinine values might have been slightly lower than if the subjects had been leading their usually active lives as were our subjects, and this may account for the higher values for the volume of the glomerular filtrate reported by Sirota and co-workers.

TABLE VII. ADDITIONAL DATA ON PATIENTS
(ALL PATIENTS ON 40 GM. PROTEIN DIET WITH EXCEPTION OF C. W. WHO WAS ON 80 GM. PROTEIN DIET)

PATIENT	DIAGNOSIS	BRIEF CLINICAL HISTORY	URINALYSIS				AUTOPSY FINDINGS
			ALB.	R.B.C.	W.B.C.	CASTS	
J. P., F, 29 Ht. 5′ 6½″ Wt. 67.7 Kg. S.A. 1.76 sq.M.	Glomerulonephritis	Albuminuria first noted Jan., 1947; since then has persistently had albumin and R.B.C. in urine. Complains of slight ankle edema since summer of 1948. Miscarriage March, 1948. B.P. normal	+++	30-40	4-5	1-3 coarsely granular	
C. S., M, 33 Ht. 5′ 9¼″ Wt. 79.7 Kg. S.A. 1.95 sq.M.	Glomerulonephritis, chronic	Albuminuria first discovered 7 years ago. Has had several recurrent episodes of edema, the last one occurring 2 years ago. Complains of occasional headaches of moderate severity. B.P. normal	+	1-3	1-3	Occasional coarsely granular	
J. W., F, 18 Ht. 5′ 5″ Wt. 74.7 Kg. S.A. 1.50 sq.M.	Glomerulonephritis	Sudden onset of edema Feb., 1947, accompanied by severe headaches and blurring of vision and dyspnea on exertion. No previous known history of kidney disease. Had severe upper respiratory tract infection in Dec., 1946. B.P. at time of onset 140/105. Asymptomatic at present, with normal B.P.	++++	30-50	0-4	5-10 granular	
E. L., F, 51 Ht. 5′ 5¼″ Wt. 77.3 Kg. S.A. 1.82 sq.M.	Hypokalemia, etiology unknown	Potassium deficiency, etiology unknown. Illness started in April, 1948, with onset of edema. Subsided with Mercuhydrin and NH_4Cl therapy. Since then has had 3 bouts of nausea and vomiting associated with marked weakness. During last attack was unable to walk. B.P. normal	+	Occ.-5	3-5	Occ. hyaline	
M. W., M, 21 Ht. 5′ 5¼″ Wt. 65.5 Kg. S.A. 1.71 sq.M.	Glomerulonephritis	Albuminuria first discovered in 1946. Has had edema on and off since Feb., 1947. In March, 1949, had episode of acute pyelitis. B.P. 190/132	++++	12-15	2-4	None	
J. L., F, 29 Ht. 5′ 7″ Wt. 73.5 Kg. S.A. 1.83 sq.M.	Glomerulonephritis, nephrotic stage	Started insidiously in October, 1948, with edema and recurrent nausea and vomiting. B.P. normal	++++	0-10	0-6	Occ. granular	
C. W., F, 30 Ht. 5′ 2″ Wt. 62.5 Kg. S.A. 1.62 sq.M.	Glomerulonephritis nephrotic stage; possible "true nephrosis"	Illness started in Dec., 1947, as edema, associated with second pregnancy. Has had repeated episodes of edema since then. Had severe headaches at start of illnes. B.P. normal then and has never been elevated	++++	0	1-3	Occ. hyaline	
B. W., M, 15 Ht. 5′ 8¾″ Wt. 58.2 Kg.	Nephritis, focal, or glomerulonephritis acute	Kidney trouble manifested as gross hematuria during hospitalization in Jan. 1949. B.P. 130/0-50. Aortic in-	+++	Numerous	5-10	Occ. red cell casts	

	sis''	of illnes. B.P. normal then and has never been elevated					
B. W., M, 15 Ht. 5′ 8¾″ Wt. 58.2 Kg. S.A. 1.69 sq.M.	Nephritis, focal, or glomerulonephritis, acute, superimposed on rheumatic heart disease	Kidney trouble manifested as gross hematuria during hospitalization in Jan., 1949. B.P. 130/0-50. Aortic insufficiency (?)	+++	Numerous	5-10	Occ. red cell casts	
W. C., M, 14 Ht. 5′ 6¼″ Wt. 60.0 Kg. S.A. 1.67 sq.M.	Glomerulonephritis	Had frequent episodes of upper respiratory tract infection in March and April, 1949, and shortly afterwards had edema and gross hematuria with headaches. Hypertension followed later. Now still edematous	+++	Many	1-3	2-4 coarsely granular	
R. L., M, 28 Ht. 5′ 7¼″ Wt. 62.7 Kg. S.A. 1.72 sq.M.	Glomerulonephritis	Albuminuria first discovered in 1942, but asymptomatic until March, 1949, when he had marked blurring of vision, nausea and severe headaches. Grade IV Keith-Waggoner retinopathy. B.P. 250/140	++++	30-40	15-20	Few coarsely granular	
D. B., F, 16 Ht. 5′ 3″ Wt. 52.7 Kg. S.A. 1.52 sq.M.	Glomerulonephritis	Started with albuminuria and edema in 1947. Complains occasionally of tiredness and weakness. Has marked anemia. Patient doing full-time high school work. B.P. 132/82	++++	30-50	5-7	Rare coarsely granular	
C. H., M, 45 Ht. 5′ 5½″ Wt. 98.6 (adm.) 78.2 (discharge) S.A. (Ideal) 1.70 sq.M.	Hypertension, ''essential,'' with hypertensive heart disease	Onset in 1943 with severe intermittent occipital headaches. Ease of fatigue and dizziness noted since April, 1948, and in Oct. had sharp but momentary precordial pain while in bed. Vision has been blurred since May, 1947. Fundus Grade IV hypertensive retinopathy, bilateral. B.P. on admission 228/140; on discharge 208/126	+++	4-5	Few	Occ. to few coarsely granular	
B. C., M, 51 Ht. 5′ 9¾″ Wt. 73.2 Kg. S.A. 1.89 sq.M.	Hypertension, ''essential''	Asymptomatic until Aug., 1949, when he complained of dyspnea and weakness. Diagnosis of arteriolar nephrosclerosis made in Sept. No edema, headaches, nor visual disturbance. No angina. B.P. 210-224 124-150	+++	None	0-2	1-2 hyaline	
R. C., M, 42 Ht. 5′ 8¼″ Wt. 67.2 Kg. S.A. 1.79 sq.M.	Glomerulonephritis, acute, hemorrhagic	Sudden onset latter part of Feb., 1949, with anasarca, gross hematuria, vomiting, headaches, blurring of vision. Still had gross hematuria on admission March, 1949. Two months after discharge urine clear grossly, although still showed R.B.C. microscopically. Follow-up studies show progressive improvement of renal function	+++ (2 mo. after disch.)	30-50	7-8	4-5 coarsely granular	

(*Continued on following page.*)

TABLE VII—CONT'D

PATIENT	DIAGNOSIS	BRIEF CLINICAL HISTORY	URINALYSIS				AUTOPSY FINDINGS
			ALB.	R.B.C.	W.B.C.	CASTS	
R. G., M, 35 Ht. 6′ 0″ Wt. 86.4 Kg. S.A. 2.07 sq.M.	Glomerulonephritis acute, hemorrhagic	Had upper respiratory tract infection accompanied by sore throat early part of May, 1949. Three weeks after onset, noticed dark "coffee-colored, smoky" urine, and had malaise, anorexia, nausea, and vomiting. Also had swelling of face and extremities which was worse in A.M. Slight dyspnea. B.P. on adm. 170/120. Now normal	+++ (on adm.)	Numerous	10-20	3-4 coarsely granular and red cell casts	
			++ (10 mo. after disch.)	50+	2-4	Occ. coarsely granular and red cell casts	
E. C., M, 41 Ht. 5′ 10½″ Wt. 71.8 Kg. S.A. 1.88 sq.M.	Glomerulonephritis	Albuminuria first discovered in 1937. Asymptomatic until 1945, when he started having headaches. First knew he had hypertension in 1947. Three weeks before admission had nausea and vomiting. Fundus shows Grade III retinopathy. B.P. 232/158	+++	10-20	Occ.	Occ. granular	
F. W., M, 26 Ht. 5′ 10¼″ Wt. 71.5 Kg. S.A. 1.87 sq.M.	Glomerulonephritis, terminal	Albuminuria first noted in 1938, followed by hypertension 3 years later. During past 3 years had transient episodes of edema. Since June, 1948, illness characterized by severe hypertension and congestive heart failure. Grade IV Keith-Waggoner retinopathy. B.P. 210/150. Patient was in and out of hospital many times during past six months. Anemia was severe, signs and symptoms of heart failure prominent, had recurrent skin infections and nose bleeds, and ascites was present to such an extent as to incite suspicion of cardiac cirrhosis of liver. On last admission Sept., 1949, patient was practically anuric during last week and finally succumbed Sept. 20	+++	Many	Few	Many coarsely granular	Markedly contracted kidneys with hardly any cortex discernible. On microscopic examination only a few hypertrophied functioning glomeruli seen. Renal arteries did not show marked changes. Cardiomegaly present. Liver enlarged and congested, but no cirrhosis. Moderate enlargement of parathyroids
B. K., M, 21	Glomerulonephr-	Acute tonsillitis in May, 1946. Nausea	+++	Loaded	Many	Many granular	Consent for

B. K., M, 21 Ht. 6′1″ Wt. 71.6 Kg. S.A. 1.92 sq.M.	Glomerulonephritis, terminal	Acute tonsillitis in May, 1946. Nausea and vomiting Aug., 1946. Gross hematuria in Sept. Six weeks later edema started. +++ albumin then. Gross hematuria disappeared Feb., 1947, but continued to gain in weight due to edema. First admitted to U. Hospital Aug., 1947. Had anasarca with marked blurring of vision. Wt. reduced from 241 to 172 lb. Severe upper respiratory tract infection 2 weeks before last adm. 10-31-49. Had increasing malaise and fatigue, progressing to orthopnea. B.P. 200/130. Died Nov. 4, 1949	+++	Loaded	Many	Many granular	Consent for autopsy not obtained
D. P., F, 37 Ht. 5′ 1″ Wt. 46.3 Kg. S.A. 1.41 sq.M.	Glomerulonephritis, terminal	Apparently in good health until June, 1944, when 5 days after onset of ''strep'' sore throat had shaking chills followed by edema. Recurrent episodes of chills and swelling. Later had headaches, followed by ease of fatigue. Three months after onset, doctor reported systolic B.P. of 210. No gross hematuria. First seen at U. Hospital in 1944, moderately edematous. Relieved of edema by low Na diet. Able to work and live practically normal life for 5 years after onset. During last six months, went progressively downhill, with marked anemia and congestive heart failure. Died in uremia Oct. 3, 1949	+++	6-10	2-5	Occ. coarsely granular	Typical findings of chronic glomerulonephritis
D. M., M, 49 Ht. 5′ 7¾″ Wt. 60.0 Kg. S.A. 1.71 sq.M.	Polycystic kidneys	Patient entered hospital in coma. Was perfectly well until 1948, when he started feeling tired. One week before admission, complained of pain over right costo-vertebral angle, had nausea and vomiting, with low-grade fever. I.V. pyelograms showed suspicious polycystic kidneys. B.P. normal during hospitalization. Patient was in marked K depletion; serum K still 3.15 meq./L. after considerable I.V. K administration. After recovery from this crisis, patient has felt well and is back working full time	+	5-6	8-10	None	

(Concluded on following page.)

TABLE VII—CONT'D

PATIENT	DIAGNOSIS	BRIEF CLINICAL HISTORY	URINALYSIS				AUTOPSY FINDINGS
			ALB.	R.B.C.	W.B.C.	CASTS	
M. K., F, 65 Ht. 5′ 1¾″ Wt. 72.0 Kg. S.A. 1.55 sq.M.	Glomerulonephritis, chronic	Admitted Feb., 1949, in semicomatose state. Had weakness since spring, 1948, associated with anorexia, nausea, vomiting. Symptoms persisted, vomiting almost every day. Wt. loss of 30-40 lb. since onset. Had known B.P. of 210 systolic since 1946. No G.U. symptoms, no history of edema, no visual disturbances. B.P. on admission 180/90. Kussmaul breathing. Discharged after 3 weeks. Patient now feels fine and able to do household chores in farm house. Present B.P. 174/92	+++	2-5	1-4	Occ. to few coarsely granular	
H. R., M, 44 Ht. 5′ 8″ Wt. 58.6 Kg. S.A. 1.69 sq.M.	Glomerulonephritis, terminal	Patient claimed to have been entirely well until April, 1948, when he felt tired and somnolent most of the time. This was followed by anorexia and attacks of severe precordial pain radiating to left arm, associated with physical activity. B.P. 170/96. Patient went progressively downhill and expired Jan. 29, 1949	+++	Occ.-6	2-10	Rare granular	Typical findings of chronic glomerulonephritis

Since creatinine determinations were done by the method of Bonsnes and Taussky, we anticipated that the creatinine clearance would give a value which was different by about ±10 per cent from the volume of the glomerular filtrate as determined by inulin clearance in patients whose filtration volume had been reduced to less than one-third of the normal value. It will be readily realized that such a discrepancy at this stage of renal damage, amounting to 5 liters more or less per twenty-four hours, is of no consequence insofar as the clinical evaluation of the patient is concerned.

It will be noted that in calculating the 24-hourly endogenous clearance of creatinine, only one serum creatinine value for a particular 24-hour period was used. Our data show that such a practice is justified, since the serum creatinine concentration varies little throughout the day when patients are receiving the small amounts of meat that are included in the 40 Gm. protein diet. Accordingly, the samples of blood were obtained during any convenient time of the day.

Twenty-four-hourly urea clearances were similarly calculated on the basis of a single determination of blood urea nitrogen from a portion of the sample of blood used for serum creatinine. It is well known that the concentration of B.U.N. is affected by any type of protein food, but our studies showed that on a 40 Gm. protein diet, the variation in B.U.N. throughout the day was trivial. For example, for patient B. R., on such a diet, the fasting value was 13.5 mg. per cent, and the hourly postprandial values were 13.3, 13.5, 13.2, and 13.1 mg. per cent.

Hourly urea clearance as usually performed are often unsatisfactory. Chief among the difficulties is inaccurate timing of the hourly urine collections, sometimes coupled with incomplete emptying of the bladder. Consider an error of five minutes in one hourly collection. This would constitute a discrepancy of 8.3 per cent. Suppose in addition to such an error in timing, there were 5 ml. of urine left in the bladder, and that the total volume for the hourly specimen was 120 milliliters. This retention of urine would in itself introduce an error of 4.2 per cent, and the two discrepancies would give an error of 12.5 per cent. Peters and Van Slyke,[21] in their discussion of urea clearance, state, "It requires, however, most meticulous attention to technical details. The rate of urine excretion must be precisely determined. The clearance test is therefore inapplicable to the subject who cannot empty his bladder completely, unless recourse is had to catheterization. . . ." This is not uncommon in elderly persons, especially in women. In addition, changes in the rate of urine formation during the test are sometimes great enough to give unsatisfactory data. Furthermore, it has been shown by Van Slyke, Alving, and Rose[22] that posture and physical activity affect significantly the urea clearance vaules in patients with less than 50 per cent of normal clearance.

On the other hand, five minutes' difference in a 24-hour collection means only 0.35 per cent error and an incomplete voiding with a residual urine of 5 ml. constitutes an error of 0.33 per cent for a usual 24-hour urine of 1,500 milliliters. Accordingly, these two latter discrepancies in the case of the 24-hour specimen cause an error of less than 1 per cent. Furthermore, clearances continuing over a 24-hour period are long enough to include variations attribu-

table to such factors as physical activity,[13] drugs,[14] emotional tension,[15] and variations in intra-abdominal[16] and venous pressure.[17] Finally, the 24-hourly collection does not interfere with the usual activity of the subjects.

The serum creatinine concentrations in all of the normal subjects were less than 1.3 mg. per cent. However, four of the patients gave serum creatinine values equally low. In these circumstances, the serum levels of creatinine are not informative regarding the question whether such patients have kidney disease. The filtrate volumes in these patients, corrected for body surface and expressed as per cent of normal, were 49, 63, 81 and 89 per cent (corrected normal range, 83 to 115 per cent). Accordingly, the measurement of the volume of the 24-hourly glomerular filtrate even in patients with normal values for serum creatinine was often an important laboratory aid in establishing the diagnosis. In the patients whose concentrations of serum creatinine ranged from 1.3 to 2.3 mg. per cent, the volume of corrected glomerular filtrate expressed as per cent of normal varied from 42 to 85, with an average of 61. Serum creatinine levels of more than 2.3 mg. per cent were invariably accompanied by filtrate volumes of less than 40 per cent of normal. When the concentration of serum creatinine rose to or above 5 mg. per cent, the corrected volume of glomerular filtrate was invariably less than 20 per cent.

SUMMARY AND CONCLUSIONS

Some of the ingested chemically pure creatinine is eliminated by tubular secretion. Most of the creatine present in muscle meat is converted to creatinine by cooking, and this then conducts itself as pure exogenous creatinine. However, low protein diets containing less than 75 Gm. of meat yield too little creatinine to affect the 24-hourly creatinine clearance. It takes forty-eight hours to eliminate the effect of high meat intakes.

The accuracy of the hourly urea clearance test is so greatly affected by improper timing and by incomplete emptying of the bladder that the values obtained in clinical practice are often unsatisfactory. Inulin clearances, while accurate, are too difficult for routine clinical use, and at best give information about the volume of the glomerular filtrate for very short intervals of time during unphysiologic conditions.

When subjects are maintained on a 40 Gm. protein diet, and when the two collections for the hourly urea clearance test are accurately timed, the latter and the 24-hourly urea and creatinine clearances, all expressed as per cent of normal, give satisfactorily comparable results. The most reliable of these tests is the 24-hourly creatinine clearance because it is less affected by inaccurate timing than the hourly urea clearance, and because it is technically easier to accomplish.

The normal range of serum creatinine in our series for men was 0.95 to 1.29 mg. per cent, and for women was 0.77 to 0.98. The average 24-hourly creatinine clearance for both men and women was 137 liters, ranging from 113 to 158 liters per twenty-four hours per 1.73 sq.M. The seventy-four clearances on twenty-two patients (corrected to body surface of 1.73 sq.M.) ranged from 122 to 0.37 liter per twenty-four hours.

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EFFECT OF CORTISONE ON EXPERIMENTAL PRODUCTION OF ARTERITIS BY PASSIVE SENSITIZATION

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INTRODUCTION

THE relationship of the adrenal cortex to immune mechanisms has been extensively studied with conflicting and inconclusive results. The report of Fox and Whitehead[1] and the studies of Dougherty, Chase, and White[2-4] indicate that the adrenal cortex is responsible for a rise in antibody titer, whereas other workers report that the titer of circulating antibody is uninfluenced by the administration of adrenal cortical extract[5] and that antibody production is actually increased in adrenalectomized animals.[6, 7] Hammond and Novak confirmed the findings of Dougherty, Chase, and White but were unable to obtain this anamnestic response when adrenal cortical extract was administered to rabbits to whom circulating lymphocytes previously had been transferred from immunized donor animals. It has also been reported that the presence of the intact adrenal gland or the administration of adrenal cortical hormone increases the resistance of animals against anaphylactic shock.[9-11]

In the study of this interrelationship with adrenal cortical steroids (compound E, cortisone) and pituitary adrenocorticotropic hormone (ACTH) varying results have been reported. These hormones have been shown to be responsible for: (1) a rise in antibody titer by means of an anamnestic response[3] or antibody liberation; (2) either no change[12, 13] or a slight diminution[14] in serum antibody; or (3) a decrease in antibody globulin following their use.[15, 16] These hormones have exerted no influence on the susceptibility of an animal to the production of anaphylactic shock[13, 17-19] but have been responsible for abolishing tuberculin skin hypersensitivity[13, 17] and shock[13] in the guinea pig. Cortisone exerts no effect on in vitro histamine liberation, the presence of histamine, and the activity of complement in the course of antigen-antibody reaction.[20]

Studies on the effect of ACTH and cortisone on tissue lesions of hypersensitivity experimentally produced in animals have been reported. Both the production of the Arthus phenomenon in the skin of sensitized rabbits[12, 13, 17, 19] and nephritis induced by the administration of nephrotoxic serum[21] appear to be unaffected by the action of these hormones. However, they are reported to exert an inhibitory effect on the cardiovascular lesions[12] and a preventive effect upon the development of arteritis[22] in experimental serum disease in rabbits actively sensitized to foreign sera.

Recently a new procedure has been reported for the experimental production of arteritis by passive sensitization in which it has been shown that the

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in vivo antigen-antibody union is the major factor involved in the production of these hypersensitive vascular lesions.[23] This report deals with a study of the effect of the administration of cortisone on the production of the lesions of allergic arteritis by passive sensitization.

EXPERIMENTAL PROCEDURE AND RESULTS

The details of the procedure for the production of vascular lesions by passive sensitization in the rabbit with a description of the lesions produced, are discussed in detail elsewhere.[23] Briefly this consists of the administration of 5 ml. of high titer rabbit antihorse serum into the marginal ear vein of a rabbit. After a one-half hour incubation period allowing for circulation, distribution, etc., of the antibodies, the animal is challenged with the intravenous administration of horse serum 1:100 in successive doses of 1 ml., 2 ml., 3 ml., 4 ml., and 5 ml. given at fifteen-minute intervals. Twenty-four hours later the animals are sacrificed by air embolism and sections of interal organs are taken for study.

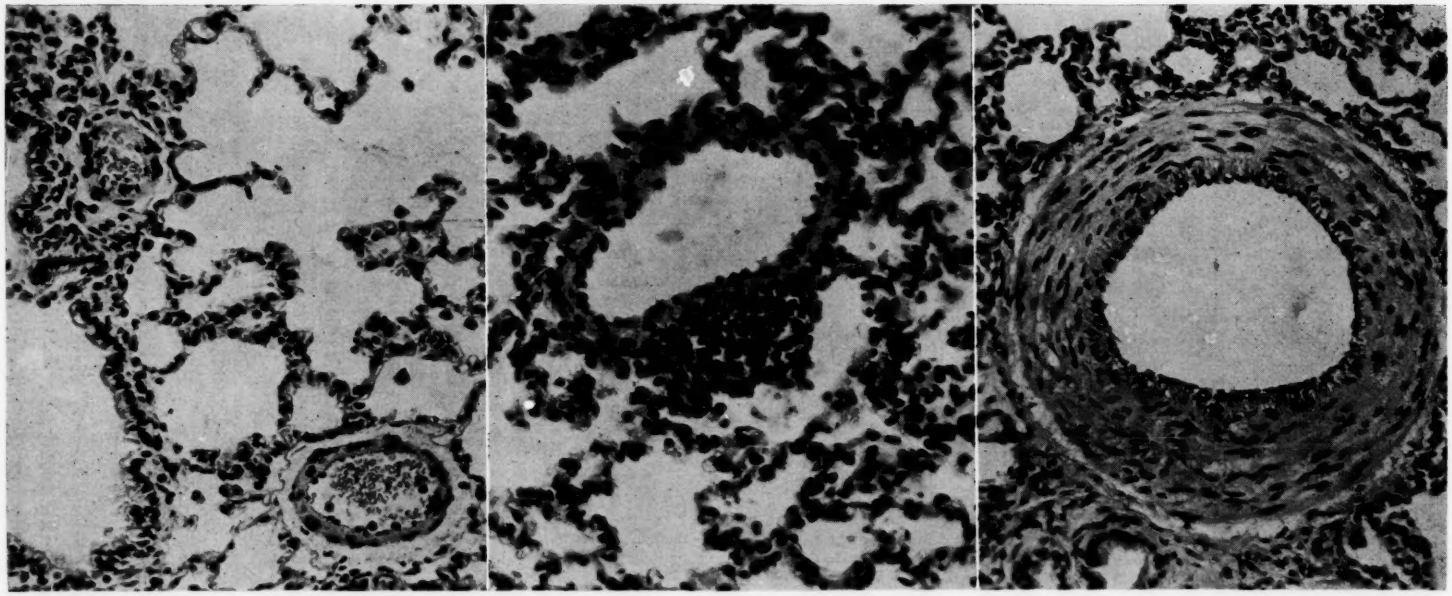
Fourteen New Zealand strain, male, albino rabbits ranging in weight from 1,500 to 2,000 grams were sensitized with rabbit antihorse serum with an antibody precipitin titer of 1:1,280, determined by titrations of antiserum dilutions against horse serum 1:5,000. The animals were then challenged with horse serum 1:100 as already indicated. Seven of the animals received cortisone acetate (Merck) 5 mg. per kilogram body weight intramuscularly every twelve hours for three doses prior to sensitization. Immediately following the third dose the animals were sensitized, challenged, and subsequently received a fourth dose of cortisone, 5 mg. per kilogram body weight eight hours later. All fourteen animals were sacrificed by air embolism twenty-four hours after sensitization. Sections were taken from the lungs, heart, liver, spleen, pancreas, adrenals, kidneys, and lymph nodes. Hematoxylin and eosin stained sections of these tissues were studied microscopically. One additional rabbit received cortisone alone according to the same schedule and dosage and was studied in a similar manner.

In the seven rabbits passively sensitized and challenged with horse serum the typical lesions of a periarteritis and panarteritis of the lung were found. The small pulmonary arteries and the pulmonary arterioles showed marked perivascular and adventitial infiltration of eosinophiles and to a lesser extent of lymphocytes and polymorphonuclear leucocytes. Eosinophiles were also found infiltrating the intima and media of the arteries and in several instances collections of eosinophiles were found within the lumina of these vessels (Fig. 1, *A* and *B*). In several sections varying degrees of hyperplasia of the endothelial cells of the intima, cloudy swelling of the muscle fibers with hydropic degeneration of the media, and some adventitial edema were found (Fig. 1, *C*). Scattered interstitial infiltration and focal accumulation of eosinophiles were seen within the alveolar walls.

In the seven rabbits that had been treated with cortisone prior to and following sensitization, pulmonary arterial lesions were much less evident and less severe in nature. Only a few of the pulmonary arterioles were affected. When lesions were observed they consisted of a light perivascular infiltration of lympho-

A. B. C.

Fig. 1.—Sections from rabbits passively sensitized to and challenged with horse serum. There is periarterial infiltration with eosinophiles and to a lesser extent with lymphocytes and polymorphonuclear leucocytes. A few eosinophiles are seen infiltrating the adventitia, media, and intima. *C* shows some intimal proliferation. (×250.)



A. *B.* *C.*

Fig. 2.—Sections from the lung of a rabbit that had received cortisone prior to and during passive sensitization and a challenging dose of horse serum. There is only a light perivascular infiltration of lymphocytes and a few polymorphonuclear leucocytes. Eosinophiles are absent. (×275.)

cytes and polymorphonuclear leucocytes. For the most part eosinophiles were absent in these periarterial cellular infiltrates in contrast to the lesions seen in the lungs of the sensitized animals that had not received cortisone. There was no evidence of eosinophilic infiltration or other pathologic changes in the media or intima of these involved vessels (Fig. 2, *A* and *B*). The pulmonary arteries were spared pathologic changes (Fig. 2, *C*).

In all of the sensitized animals, both untreated and treated with cortisone, the heart, liver, spleen, kidneys, pancreas, and adrenals were microscopically normal.

Microscopic study of the sections of lung, heart, liver, spleen, kidneys, pancreas, and adrenal of the nonsensitized rabbit who had received cortisone alone revealed no significant abnormality.

DISCUSSION

The exact relationship of the adrenal cortex to the immune response is not clear on the basis of reported animal experiments and clinical studies of allergic diseases. However, it does seem that adrenal cortical steroids exert a definite effect on immune mechanisms. According to Fischel[19] it appears that cortisone and ACTH do not alter the union of antigen and antibody. Among suggestions advanced to explain their effect on immune processes are the following: (1) an inhibition or diminution of antibody production,[19] (2) a degradation of antibody protein,[17] (3) production of lymphopenia and involution of lymphoid tissue[3, 24] a suspected site of antibody production and transport, or (4) perhaps a suppression or decrease in the reactivity of mesenchymal tissue.[25]

It was felt that the level at which this effect was exerted might be studied profitably by noting the effects of cortisone in the procedure of passive sensitization. Heretofore the effects of cortisone and ACTH have been studied on the experimentally produced vascular lesions in actively sensitized animals employing large doses of foreign serum. A suppressive effect on these lesions was noted,[12, 22] but in this type of experiment two factors in the immune mechanism come into play: (1) antibody production and (2) antigen-antibody union. Thus the effect of the hormone on each individual process cannot be distinguished. However, by using the procedure of passive sensitization, as here employed, the important mechanism is that of an in vivo antigen-antibody union, and any effect that cortisone might exert on lesions thus produced can be attributed to its action at this point in the immune process.

SUMMARY

Fourteen albino rabbits of the same strain and approximate age and weight were passively sensitized to and challenged with horse serum according to a procedure favoring the production of acute lesions of periarteritis and panarteritis of the pulmonary arteries and arterioles.

Cortisone was administered to seven of the animals for twenty-four hour periods prior to and following sensitization.

All of the sensitized animals that had not received cortisone developed the typical lesions of panarteritis and periarterial infiltration of eosinophiles and to a lesser extent of lymphocytes and polymorphonuclear leucocytes, and eosinophilic infiltration into the media and intima of the small pulmonary arteries and pulmonary arterioles.

Those sensitized animals that had also received cortisone exhibited less extensive and modified type of lesions consisting of a light periarterial infiltration of lymphocytes and polymorphonuclear leucocytes of the pulmonary arterioles. Eosinophiles were only rarely seen in the lesions of this group of animals.

The results indicate that when cortisone was administered prior to and following passive sensitization to horse serum the lesions of experimentally produced arteritis in rabbits appear to be modified though not completely suppressed. The lesions of panarteritis of the pulmonary arteries were not found and those of a periarteritis appeared to be attenuated. The reason for this is not clear although it is thought that perhaps cortisone exerts an effect upon the in vivo union of antigen-antibody or may influence the tissue response evoked by antigen-antibody reaction.

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THE ACTH-LIKE EFFECT OF FEVER IN MAN

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INVESTIGATIONS of the effects on malignant hypertension of an endogenous component of *Proteus vulgaris*,* hereafter referred to as Pv Pyromen, disclosed a marked drop in circulating eosinophils which occurred regularly four to eight hours after the intravenous injection. This phenomenon has been reported several times in patients after intravenous administration of typhoid vaccine[1, 2, 3, 5] and its products[4] and recently by Randolph and Rollins[6] in a group of allergic patients treated with Pv Pyromen. Since eosinopenia occurs in man after the administration of adrenocorticotropic hormone (ACTH),[7, 8, 9] these observations suggested that fever-producing agents might stimulate production of endogenous ACTH. Selye[10] has suggested that fever therapy mobilizes the endocrine defense mechanisms during the general adaptation syndrome. Windle and associates[11] have reported changes in the adrenal glands of animals following Pv Pyromen administration which simulate those produced by ACTH. In an effort to determine if the eosinopenia observed after induced fever was due to the liberation of ACTH, the following observations were made.

MATERIALS AND METHODS

The subjects selected were twenty-seven afebrile patients with the diseases shown in Table I. The patients with hypertension were assumed to have normally functioning pituitary and adrenal glands, and in the remainder the response to the "epinephrine tests"[12] indicated adequacy of the pituitary-adrenocortical axis. Fever was induced in six subjects by intravenous typhoid vaccine, in six by a warm, moist air fever cabinet, and in fifteen by the intravenous administration of Pv Pyromen. Two of the patients given typhoid vaccine had previously had typhoid immunization. Uric acid and creatinine determinations were made on urine collected at two-hour intervals during the day and on a fourteen-hour overnight specimen. Control blood counts and uric acid:creatinine ratios were determined on fourteen of the twenty-seven subjects utilizing identical methods for a twenty-four hour control period prior to induction of fever. In five patients, after observations for four to seven control days, corticosteroid excretion in the urine after fever induced by Pv Pyromen was measured by the method of Ashby.[13] Aspirin was given to two patients before and after injection of Pv Pyromen. Dibenamine and Chlor-Trimeton,† an antihistamine compound, were used similarly in two patients. The same observations on the leucocyte count and excretion of uric acid and creatinine were made.

Circulating leucocytes were counted by the method of Randolph.[14] Urinary uric acid and creatinine were measured by modification of Folin's[15, 16] methods. Body temperature was

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*Pyromen, Baxter Laboratories, Inc.

†Chlor-Trimeton. 1-parachlorophenyl-1-(2-pyridyl)-3-dimethyl-aminopropane maleate.

TABLE I. CLASSIFICATION OF PATIENTS AND TYPE OF FEVER

	TYPHOID VACCINE	FEVER CABINET	PV PYROMEN	CONTROLS
No disease	1			1
Arteriosclerosis obliterans	1			1
Convalescent poliomyelitis	2			2
Psoriasis	1		1	
Diabetes mellitus	1		1	
CNS syphilis		6		5
Rheumatoid arthritis			5	2
Chronic glomerulonephritis			2	
Hypertension			4	3
Myxedema (controlled)			1	
Hyperthyroidism (controlled)			1	
	6	6	15	14

recorded rectally at two-hour intervals during febrile periods. In this study dosages of 25 to 100 gamma of Pv Pyromen and 15 to 100 million typhoid organisms were used. None of the subjects had had intravenous fever therapy previously.

RESULTS

Fever produced by these three methods caused a marked and significant drop in eosinophils as shown in Fig. 1. With Pv Pyromen, the maximal fall in circulating eosinophils occurred at four hours, the time of the highest fever. They remained at a significantly low level for the remaining four hours of the observation period. Eosinophil counts after eight hours were not done on the entire group but observations on six patients in this group and on six others treated similarly disclosed a rise up to or above pretreatment levels fourteen to twenty-four hours after therapy was started. With typhoid vaccine, however, the maximal drop in eosinophils occurred in twenty-four hours, ten hours after the highest temperature. The count had not risen to pretreatment levels at the end of the observation period. The maximal eosinopenia in the fever cabinet group occurred at eight hours, four hours after the highest temperature. Thus the fall in eosinophils was roughly of the same degree in all three groups; the only difference was in the rapidity of the fall. On the other hand, eosinophil counts on the control group had risen 60 per cent above initial values by the end of fourteen hours.

The response of the total white blood count to the three types of fever is shown in Fig. 2. A marked rise occurred in each instance, the least marked in the typhoid vaccine group. The maximal rise in the fever cabinet and Pv Pyromen groups occurred four hours after the peak of fever, while in the typhoid vaccine group the maximal rise preceded the highest temperature by ten hours. The white blood count had returned to normal levels fourteen hours after fever induced by the fever cabinet. In six individuals in the Pv Pyromen group and in six others treated similarly it was observed that the leucocyte count was normal at twenty-four hours. In the typhoid vaccine group, the white blood count fell below control levels at the peak of the febrile response at fourteen hours and remained low until the end of the period of observation. The leucocyte response of the two patients previously immunized to typhoid vaccine was essentially the same as in those not immunized.

There was a sharp and rapid rise in the proportion of polymorphonuclear cells in the Pv Pyromen group as seen in Fig. 3. The greatest rise occurred two hours after the peak of fever. The polymorphonuclear:mononuclear ratio was returning toward normal at the end of the period of observation, two hours later. Likewise, there was an increase in the polymorphonuclear cells in every patient in the typhoid vaccine and fever cabinet groups although in neither was the average increase as great as with Pv Pyromen. The relative increase in polymorphonuclear leucocytes persisted in the typhoid group for twenty-six hours following treatment. Obscured by the polymorphonuclear: mononuclear ratio in Fig. 3 is the fact that the smallest absolute drop in mononuclear cells occurred in the fever cabinet group.

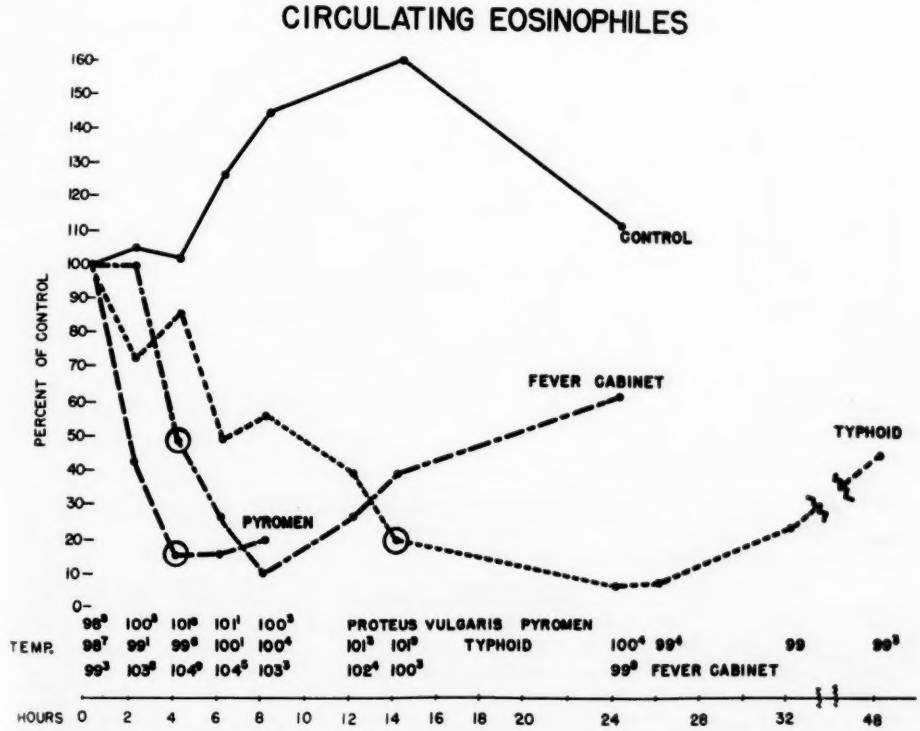

Fig. 1.—Eosinophile response to fever produced by Pv Pyromen, typhoid vaccine, and fever cabinet. In this and in Figs. 2, 3, and 4 the deviation from the control or pretreatment level expressed as 100 per cent is shown on the ordinate. Averages of each group of subjects are used. Temperature response and hours after induction of fever are represented on the abscissa.

Average values for the uric acid:creatinine ratio are given in Fig. 4. In the control group the ratio decreased as the day wore on. There was no such trend in the Pv Pyromen group. While there was a trend toward a rise of the ratio in the typhoid vaccine and fever cabinet groups it was statistically insignificant.

Fig. 5 shows the values for corticosteroid excretion in the urine before and after Pv Pyromen induced fever. There was no rise.

In two patients the expected rise in temperature was prevented by aspirin administered prior to and after a large dose of Pv Pyromen. The same blood leucocyte changes took place in the absence of fever. In addition, two patients were given Chlor-Trimeton and two others Dibenamine before, during, and after fever produced by Pv Pyromen without influencing the expected leucocyte changes.

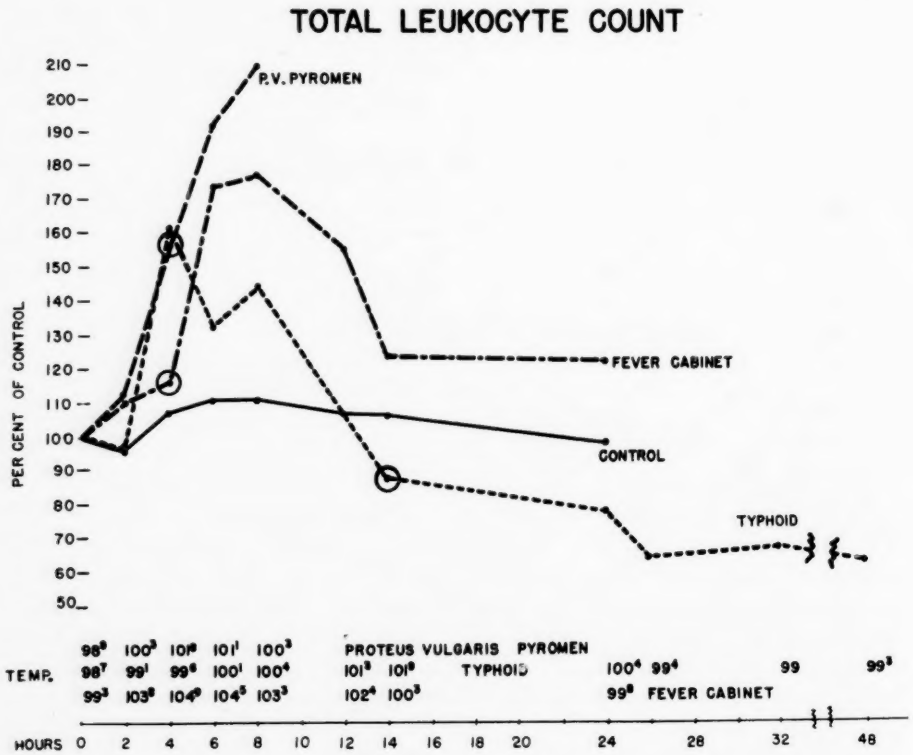

Fig. 2.—Response of total leucocyte count after fever induced by Pv Pyromen, typhoid vaccine, and fever cabinet.

DISCUSSION

Forsham, Thorn, Prunty, and Hills[8] demonstrated that a single injection of ACTH resulted in a moderate polymorphonuclear leucocytosis, marked eosinopenia, and a less consistent fall in lymphocytes four hours after administration. There was also a marked increase in the uric acid:creatinine ratio at the same time which averaged 78 per cent in fifty persons. Sayers and associates[17] have demonstrated a rise in corticosteroid excretion (C_2O carboxyl group) within the forty-eight hours following intravenous administration of a single large dose of ACTH.

Our results indicate that the blood leucocyte changes following fever induced by three methods simulate those following administration of ACTH.

Three differences stand out. First, the degree of leucocytosis, the rise of polymorphonuclear leucocytes, and the fall of mononuclear cells are greater after induced fever than has been reported in persons receiving ACTH. Second, the eosinopenia, especially in the case of the typhoid vaccine group, comes on later than that following ACTH. Third, the leucopenia which lasts for forty-eight hours following typhoid vaccine is distinctive and is not seen after Pv Pyromen, fever cabinet, or ACTH. It should be noted that the leucocyte response following pyrexia produced by fever cabinet most nearly simulates the changes seen after ACTH.

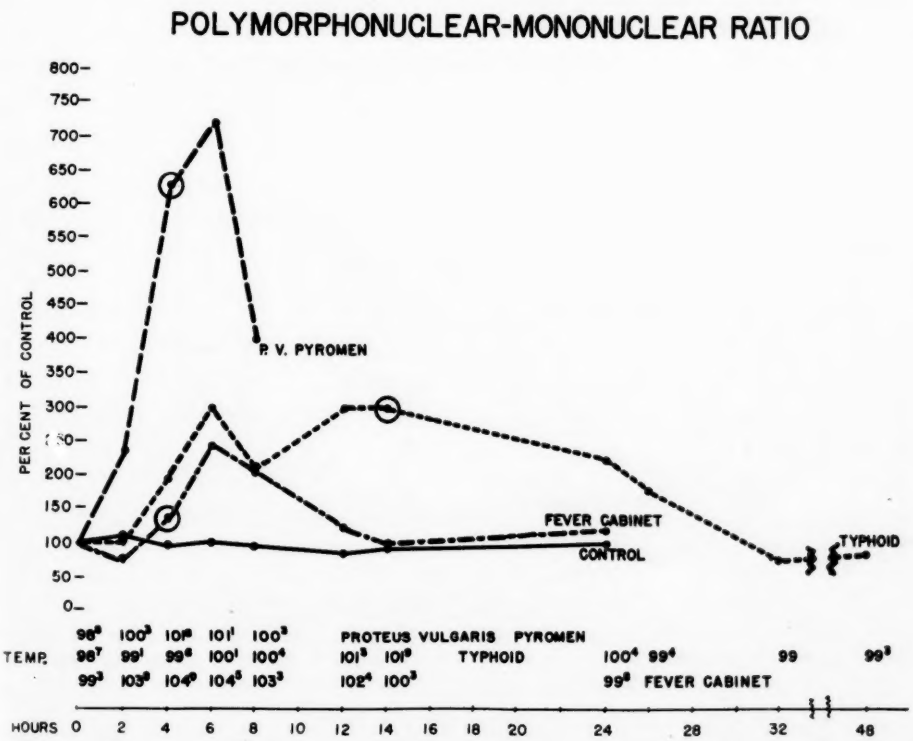

Fig. 3.—The polymorphonuclear:mononuclear ratio after fever induced by Pv Pyromen, typhoid vaccine, and fever cabinet.

The differences in the leucocyte response between these fever-producing agents and ACTH deserve comment. The immmunizing property of typhoid vaccine[18] and its purified pyrogenic products[19] has long been known. This property, which may or may not operate through the anterior pituitary, plus a toxic one which probably is not mediated through the anterior pituitary[20] might account for the initial absolute drop in mononuclear cells and the prolonged leucopenia. Lewis and Page[20] have shown that in adrenalectomized rats marked lymphopenia may occur following administration of lethal doses of typhoid vaccine in the absence of hormones from the adrenal cortex.

Altschule, Parkhurst, and Promisel[3] demonstrated that the leucopenia which regularly follows the initial dose of typhoid vaccine does not occur after later doses. They believe that this change is a manifestation of immunity of the organism to this substance and the leucopenia is the result of widespread lymph cell destructionn which follows the initial dose of typhoid vaccine in animals. The neutrophilia which develops following typhoid vaccine apparently is largely dependent on the presence of adrenal steroids[20] and may simply be due to activation of the pituitary-adrenal cortical axis by this stress. Since it is higher than that seen after ACTH, the possibility of another mechanism must be considered.

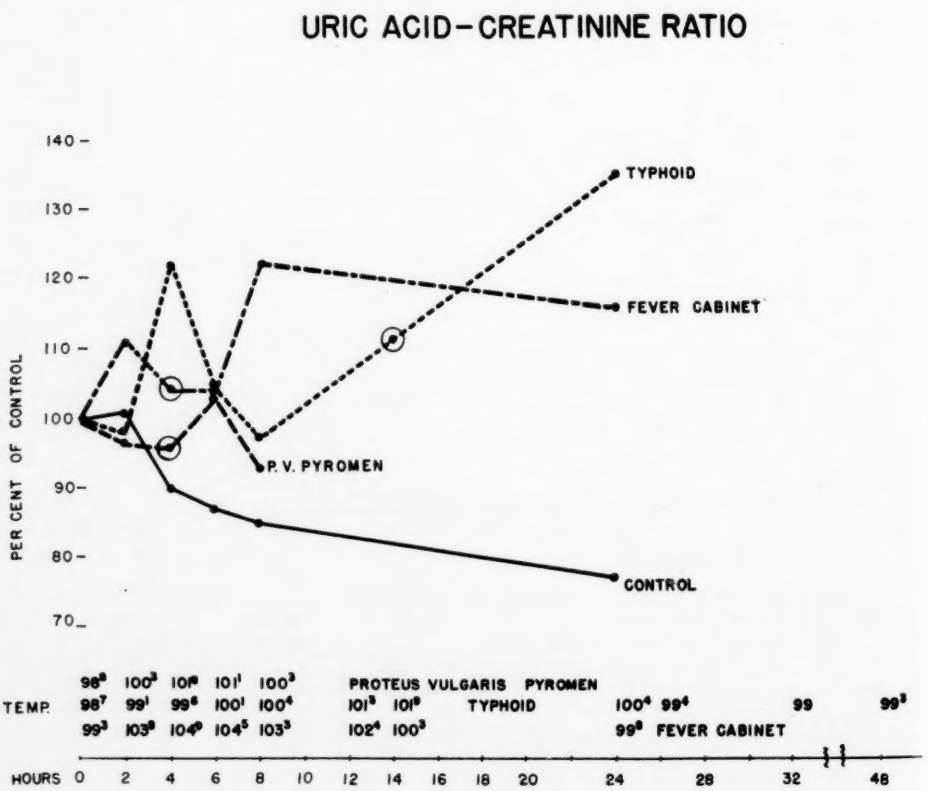

Fig. 4.—Uric acid:creatinine ratio after fever induced by Pv Pyromen, typhoid vaccine, and fever cabinet.

Probably the same immunologic and toxic factors are responsible for the differences in the leucocyte response after Pv Pyromen and ACTH. It is known that both animals[21] and man[27] slowly develop tolerance to Pv Pyromen and it causes a low degree of toxicity in animals.[21] Recently, Wells[22] has suggested that the eosinopenia observed in animals following Pv Pyromen is not a function of the pituitary-adrenocortical axis. In adrenalectomized dogs, either

untreated or treated with desoxycorticosterone acetate, he has found a drop in eosinophils with Pv Pyromen which is as great as in a group of normal animals so treated, whereas this was not so with ACTH.

Since adaptation and immunity do not develop in man following fever cabinet therapy,[23] the leucocyte response from this stress would be expected to mirror more closely other nonspecific stresses or the administration of ACTH. This is borne out by our study. There is less of a mononuclear drop than in the other types of fever and although there is leucocytosis, is is not as profound as that following Pv Pyromen. This degree of leucocytosis may be seen in some individuals following administration of small doses of ACTH. It is believed that this type of fever represents a relatively pure response, free from the many hematologic side effects seen in the other forms of fever studied.

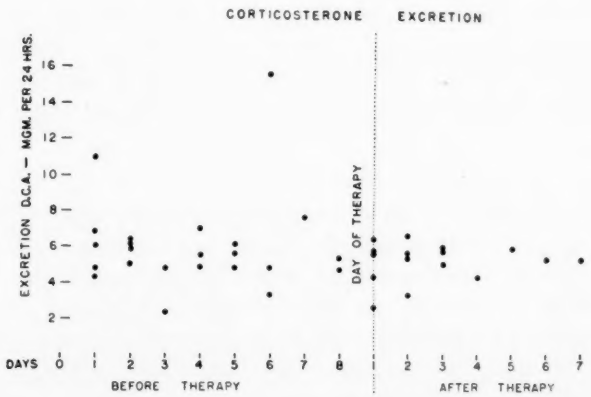

Fig. 5.—The excretion of urinary corticosteroids in five patients after Pv Pyromen.

In none of the groups did fever cause a consistent increase in the uric acid:creatinine ratio such as that which follows administration of ACTH. The data demonstrate a trend toward such an increase and this is in sharp contrast to the diurnal changes in the controls followed without therapy. Altschule, Parkhurst, and Promisel[3] recently suggested that the variable increases in uric acid:creatinine ratio that they observed following typhoid vaccine fever might be explained by the effects of the fever itself on metabolic processes, renal function, and dehydration when fever rose to high levels. These factors may account for the variations in this ratio which we found.

The absence of increased urinary excretion of corticosteroids following fever induced by Pv Pyromen is not what one would expect if large doses of ACTH were liberated by such fever. We have no evidence, however, that urinary corticosteroids are increased following a single injection of a small dose of ACTH. The failure to demonstrate increased urinary corticosteroids after Pv Pyromen does not imply that there has not been an increased production. Corcoran and Page[24] pointed out that the renal threshold for these substances probably is high and that only after great rises in blood levels do

they appear in the urine in increased quantities. On the other hand, using chemical methods different from ours, Corcoran and Page[24] have shown a rise in corticoids following fever induced in patients with malignant hypertension. Perhaps our acute experiments did not provide the stimulus for production of large amounts of corticosteroids and this may, in turn, explain the lack of increase of these substances in the urine.

Beeson[25, 26] has postulated from work in rabbits that fever from pyrogenic agents is dependent on a nonspecific substance liberated from damaged cells prior to phagocytosis of pyrogenic materials by the reticuloendothelial system. This mechanism may exist in man.[27] Beeson has postulated also that this substance acting on hypothalamic heat centers changes heat-regulating mechanisms of the body. In an effort to determine whether this nonspecific substance was histamine or epinephrine, we gave two groups of two persons each Chlor-Trimeton and Dibenamine after fever was produced by Pv Pyromen. Fever and the expected leucocyte change still occurred. Similarly, two subjects given aspirin had the characteristic leucocyte response to Pv Pyromen. This suggests that this pyrogenic agent may cause these blood changes without fever.

CONCLUSIONS

The blood leucocyte response following fever induced by fever cabinet, typhoid vaccine, and Pv Pyromen simulates that seen after administration of ACTH. The uric acid:creatinine ratio and corticosteroid excretion were not significantly altered, contrary to what would be expected if the leucocyte response was caused by liberation of adrenocorticotropic hormone. The possibility exists that the stress, acting over a relatively short time, was sufficient to alter the blood picture but not the excretion of uric acid, creatinine, or corticosteroids. We have presented evidence which suggests that in persons with normal pituitary glands fever and pyrogenic substances cause the release of small amounts of ACTH. Additional evidence is necessary to prove this point.

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THE EFFECT OF ACTH AND CORTISONE ON PNEUMOCOCCAL AND INFLUENZA VIRAL INFECTIONS IN THE WHITE MOUSE

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WHEN pituitary adrenocorticotropic hormone (ACTH) was administered to patients with pneumococcal or primary atypical pneumonia, prompt defervescence and relief of symptoms resulted. In the pneumococcal pneumonias this symptomatic improvement occurred despite the persistence of bacteremia and of rusty sputum containing viable and unphagocytosed pneumococci.[1] Although empyema appeared in one patient with Type 1 pneumococcal pneumonia, and fever returned in another with primary atypical pneumonia after the ACTH was withdrawn, the clinical changes which occurred while the hormone was being administered suggested that some of the mechanisms concerned with resistance to infection might be regulated by the adrenal cortical hormones. In an attempt to elucidate such mechanisms, the effects of ACTH and cortisone on pneumococcal infections in immune and nonimmune mice and on influenza viral infections in mice were investigated. A preliminary summary of the findings has been given elsewhere.[2]

METHODS AND MATERIALS

Experimental Animals.—White Swiss mice of the Tumblebrook farm strain were used throughout. In experiments with the influenza virus, these were male mice weighing 20 to 25 grams except where otherwise noted. The reasons for using large mice are discussed below. In the study of pneumococcal infections adult male mice were used initially, but adult female mice were also used later with no apparent difference in the results.

Hormones.—ACTH† was diluted in saline so that 0.5 ml. contained the desired dose. The usual dose was 2.5 mg. of ACTH given intraperitoneally every twelve hours. This dosage schedule has been found to produce sustained eosinopenia in this strain of mice for four to six days.[3] ACTH was administered for forty-eight to seventy-two hours in experiments dealing with pneumococcal infection, and for six to seven days in experiments dealing with influenzal infection.

The first dose of ACTH was given four hours before inoculation with the infectious agent. Mice infected with pneumococci received subsequent injections of ACTH from separate needles for each mouse, in order to minimize the likelihood of cross-infection. In some experiments control mice received 0.5 ml. of saline every twelve hours, and separate needles were also used for these injections. It was not thought desirable to utilize saline-treated controls in all experiments inasmuch as the injection of saline into mice itself induces eosinopenia, presumably as a result of adrenocortical stimulation.[3, 4] However, in several parallel experiments no difference was observed between controls treated with saline and controls not so treated.

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†Kindly supplied by Dr. J. R. Mote of the Armour Laboratories. The dosages of ACTH are given in terms of the LA-1A standard.

Cortisone* was used as a saline suspension containing 25 mg. of cortisone acetate per milliliter plus suspending agents. Preliminary experiments showed that an initial dose of 10 mg. given subcutaneously followed by daily doses of 5 mg. for five more days maintained eosinopenia in mice during the six days when the hormone was being administered. It is essential that intraperitoneal injections of this hormone be avoided. Mice receiving the cortisone by this route frequently became hyperpneic, immobile, and showed evidence of peripheral vascular collapse, from which they recovered spontaneously in about thirty minutes.

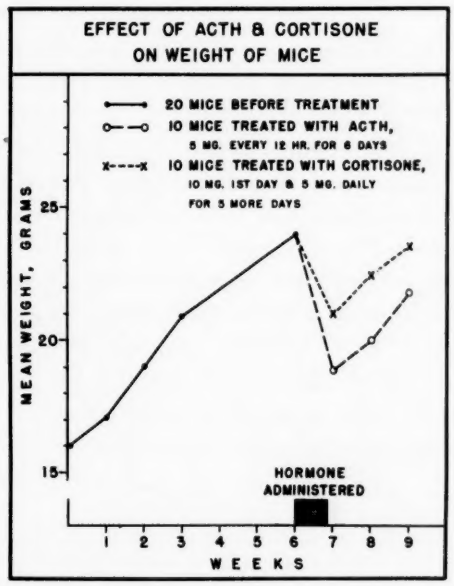

Fig. 1.

The administration of the hormones appeared to be well tolerated, but the mice uniformly lost weight, probably as a consequence of the loss of nitrogen which is induced by ACTH and cortisone. This decrease in weight amounts to as much as 20 to 25 per cent of the total body weight after but six days of administration of ACTH. For example, twenty male mice were weighed at regular intervals and given food and water ad libitum. After the rate of growth had been established, 2.5 mg. of ACTH were administered every twelve hours for six days to ten of them. The mice were weighed again twenty-four hours after the last dose of hormone, this time being chosen in order to permit subsidence of the edema which may accumulate during the time of administration of ACTH. The mean weights of the mice decreased from 23.8 to 18.2 grams in six days, but the mice quickly regained most of the lost weight after the hormone was discontinued (Fig. 1). Cortisone, administered to the other ten mice, induced a mean loss of 3 grams in six days and the initial rate of growth was resumed promptly after the administration of steroid was stopped.

Infectious Agents.—Mouse virulent Type 2 pneumococci were used; the minimal lethal dose was 1 to 3 organisms. The desired dilutions were made in broth from an eighteen-hour blood broth culture and 0.5 ml. was injected intraperitoneally into each mouse. Colony counts made with 0.5 ml. of the 10^{-7} dilution in blood agar plates yielded 3 to 10 colonies.

In some experiments, mice were partially protected with specific antipneumococcal rabbit serum. Previous titrations were performed to determine the dilution of antiserum which, when mixed with an equal volume of bacterial suspension and injected intraperitoneally,

*Purchased from Merck & Co., Inc.

would protect approximately 50 per cent of the mice from approximately 50,000 pneumococci. This amount of antiserum was then mixed with equal volumes of varying dilutions of pneumococci containing 10^3 to 10^7 pneumococci per milliliter and 0.5 ml. of the mixture was injected intraperitoneally as soon as possible after the dilutions were made.

The PR8 strain of influenza A virus used in these studies was first enhanced in virulence for mice by serial intranasal passages. Infection was induced by intranasal instillation of 0.05 ml. of a 10 per cent suspension of infected mouse lung into lightly anesthetized mice.

EXPERIMENTAL

Effect of ACTH and Cortisone on Pneumococcal Infection in Unprotected Mice.—Neither ACTH nor cortisone protected mice against small doses of Type 2 pneumococci as shown in Table I. In another experiment, four times as much ACTH was given every twelve hours with substantially the same results. There was a tendency, in repeated experiments, for mice receiving ACTH to survive for a few hours longer than control mice, although those which lived longer were obviously ill and usually moribund at the time that the control mice succumbed. Necropsies were performed on many of the mice dying in these experiments. The adrenal glands of infected mice which had received ACTH were uniformly larger than those of infected mice which had not received ACTH. There was no gross indication that ACTH might have failed to act because of adrenal insufficiency induced by the fatal infection. Mice receiving cortisone tended, in repeated experiments, to succumb a few hours sooner than control mice.

TABLE I. EFFECT OF ACTH AND CORTISONE ON PNEUMOCOCCAL INFECTION IN MICE

TREATMENT	ACTH*				CORTISONE†			CONTROL			
DILUTION OF CULTURE	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Mouse											
1	D20‡	D20	D30	D36	D18	D22	D22	D18	D18	D20	D24
2	D20	D24	D36	D36	D18	D22	D22	D18	D18	D24	D24
3	D24	D24	D36	D36	D18	D22	D22	D18	D20	D24	D30
4	D24	D24	D36	D36	D18	D22	D24	D18	D20	D24	D30
5	D24	D24	D36	D36	D18	D22	D24	D18	D20	D24	D30
6	D24	D30	D36	D36	D18	D24	D24	D18	D20	D24	D30
7	D30	D30	D36	D36	D18	D24	D24	D20	D20	D24	D36
8	D30	D30	D36	D48	D18	D24	D24	D20	D24	D30	D36
9	D36	D30	D36	D48	D18	D24	D24	D20	D24	D30	D36
10	D36	D36	D36	D48	D20	D24	D24	D20	D24	D30	S

*ACTH: 2.5 mg intraperitoneally every twelve hours.
†Cortisone: 10 mg. initially, then 5 mg. daily subcutaneously.
‡D = Died; the number represents hours after infection when death occurred.
S = Survived seven days.

Effect of ACTH and Cortisone on Pneumococcal Infection in Passively Protected Mice.—The strain of pneumococcus which was used in these experiments was so highly virulent for the mice that the animals could be considered, for practical purposes, to have no native immunity to the microorganisms. Since it was conceivable that ACTH might stimulate host reponses associated with immunity but fail to exert any effect in the absence of immunity, the mice were given sufficient antiserum to protect them against a large challenge dose. Such

immunized mice were then given doses of pneumococci large enough to overcome the immunity conferred upon the animals; the effect of treatment of such mice with ACTH and cortisone is shown in Table II.

TABLE II. EFFECT OF ACTH AND CORTISONE ON PNEUMOCOCCAL INFECTION IN PASSIVELY IMMUNIZED MICE**

TREATMENT	ACTH*			CONTROL		
DILUTION OF CULTURE	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
Mouse						
1	D17‡	D28	S	D17	D27	S
2	D17	D35	S	D17	D29	S
3	D17	D42	S	D18	D30	S
4	D24	D52	S	D21	D37	S
5	D24	D67	S	D24	D37	S
6	D24	S	S	D27	D44	S
7	D24	S	S	D29	S	S
8	D24	S	S	D30	S	S
9	D40	S	S	D35	S	S
10	D40	S	S	D35	S	S
TREATMENT	CORTISONE†			CONTROL		
DILUTION OF CULTURE	10^{-2}	10^{-3}	10^{-4}	10^{-2}	10^{-3}	10^{-4}
Mouse						
1	D20	D20	D32	D20	D32	D32
2	D20	D32	D32	D20	D32	S
3	D20	D32	D32	D20	S	S
4	D20	D32	D32	D20	S	S
5	D20	D32	S	D20	S	S
6	D20	D32	S	D20	S	S
7	D20	D60	S	D21	S	S
8	D20	D60	S	D28	S	S
9	D20	S	S	D28	S	S
10	D20	S	S	D28	S	S

**Each mouse received a dose of specific antipneumococcal rabbit serum sufficient to protect approximately half of the mice injected with 50,000 pneumococci

*ACTH: 2.5 mg. intraperitoneally every twelve hours.

†Cortisone: 10 mg. initially, then 5 mg. daily, subcutaneously.

‡D = Died; the number represents hours after injection when death occurred.

S = Survived seven days.

Neither ACTH nor cortisone increased the survival time of partially immunized animals challenged with lethal doses of pneumococci. The experiment was repeated using twice as much ACTH per mouse (5.0 mg. every twelve hours) with the same results. As in the previous experiments, the administration of cortisone to the protected mice was associated with more rapid death, and, in this instance, with a slightly greater mortality than in control animals.

Effect of ACTH and Cortisone on Influenza Viral Infections in Mice.— Resistance of mice to fatal infection with the virus of influenza increases with the age of the host.[5] The initial experiments with the PR8 strain of influenza virus were therefore conducted using male mice weighing 20 to 25 grams. In this way, animals with maximal "natural" immunity to the virus could be used, in order to increase the likelihood that an effect of the adrenocortical hormones on resistance to the virus might be observed. Table III shows that 2.5 mg. of ACTH given every twelve hours did not significantly alter the survival rate of large mice infected with the PR8 strain of influenza virus.

Gross examination of the pulmonary lesions in surviving mice was performed twelve days after the beginning of the experiment and the extent of residual consolidation was estimated. There were no significant differences between treated and control mice using either death or the presence of pulmonary consolidation as end points. The experiment was repeated using smaller mice, weighing 15 to 18 grams, with substantially the same results. When the dose of ACTH was doubled, there was no change in the numbers of mice surviving infection. Cortisone not only failed to protect mice, but seemed, as in the pneumococcal experiments, to be associated with a higher mortality rate in mice treated with this hormone.

TABLE III. EFFECT OF ACTH AND CORTISONE ON INFECTION WITH PR8 STRAIN OF INFLUENZA A VIRUS IN MICE

TREATMENT	ACTH*				CONTROL			
DILUTION OF VIRUS	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Mouse								
1	D4‡	D6	D9	S+++	D4	D3	D3	S+++
2	D4	D6	D11	S++	D5	D5	S++++	S++
3	D4	D6	S++++	S+	D5	D6	S++	S++
4	D5	D7	S+++	S+	D5	D6	S++	S++
5	D5	D7	S+++	S+	D5	D7	S++	S++
6	D5	D7	S+++	S+	D5	D7	S++	S++
7	D5	D11	S+++	S0	D5	D7	S+	S++
8	D7	D11	S+++	S0	D6	S++	S++	S++
9	D9	S+++	S++	S0	D11	S+	S0	S0
10	D11	S+++	S0	S0	S++	S0	S0	S0

TREATMENT	CORTISONE†			CONTROL		
DILUTION OF VIRUS	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-4}	10^{-5}
Mouse						
1	D3	D3	D5	D4	D5	D8
2	D3	D3	D5	D4	D5	D8
3	D3	D4	D5	D4	D5	D8
4	D3	D4	D5	D5	D5	D8
5	D4	D4	D5	D5	D5	D9
6	D4	D4	D6	D5	D6	D10
7	D4	D4	D7	D5	D6	S++
8	D4	D5	D7	D5	S+++	S++
9	D4	D5	D7	D6	S+++	S+
10	D5	D5	D8	D6	S+++	S+

*ACTH: 2.5 mg. intraperitoneally every twelve hours.

†Cortisone: 10 mg. initially, then 5 mg. daily, subcutaneously.

‡D = Died; the number represents the days after infection when death occurred.

S = Survived twelve days. The survivors were necropsied and the extent of consolidation of the lungs was estimated as ++++, 100 per cent; ++, 50 per cent, etc. 0 = No lesions.

Necropsies performed on the mice succumbing to the infection disclosed that mice receiving ACTH had larger adrenal glands which appeared to contain more lipid than those of control mice.

One group of mice was infected with a 10^{-5} dilution of infected mouse lung (approximately 6 LD_{50}), and the mice were given ACTH as before. Each day thereafter for six days four treated and four control mice were sacrificed and their lungs removed. A 10 per cent suspension of the pooled lungs from each group was prepared in serum broth and stored at –72° C. until completion of the experiment. Hemagglutinin titers of these suspensions were then compared

using the Salk technique, and it was found that ACTH did not alter significantly the rate of formation of hemagglutinins for chicken red blood cells in infected mouse lungs (Fig. 2). The infectivities of the suspensions of mouse lung when injected intra-allantoically in embryonated eggs were also compared and again there were no significant differences in the titers of lungs from mice receiving ACTH as compared with those obtained with lungs from control infected mice which had not received the hormone. ACTH, therefore, did not exert any significant effect on the rate of multiplication of the PR8 strain of influenza virus in the murine lung.

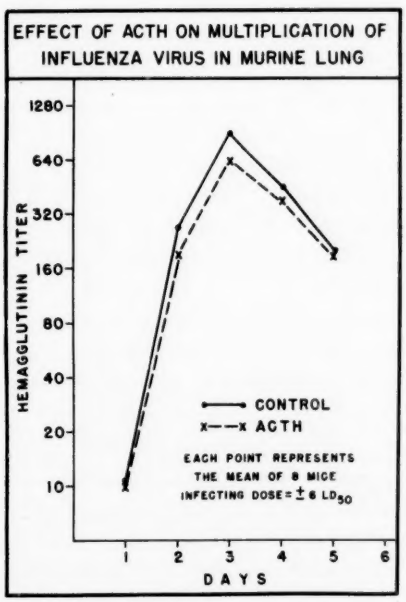

Fig. 2.

DISCUSSION

It has been amply demonstrated that absence of the adrenal cortices renders the animal host more susceptible to the effects of infections and to a wide variety of toxins.[6, 7] Adrenal cortical extracts, administered to adrenalectomized animals in sufficient amounts, restore, more or less completely, the resistance to toxic and infectious agents.[6, 7] However, attempts to increase the resistance of intact animals to various toxic and infectious agents by the use of adrenocortical extracts have led to conflicting and variable results.[6, 8, 9]

The studies presented in this paper demonstrate that neither ACTH nor cortisone, in large doses, increased the survival rate of mice infected with pneumococci or with influenza virus. Similar failures to protect mice against streptococcal infection and rats against pneumococcal infection[10] and to alter influenzal infection[11] using ACTH or cortisone have been reported. ACTH has not altered the clinical course of poliomyelitis.[12] Furthermore, ACTH has failed to protect

mice and rats against the acute toxic action of concentrated influenza virus, and did not alter the resistance of mice to the toxic action of concentrated suspensions of rickettsiae.[13]

The treatment of pneumococcal infection with adrenocortical extracts has resulted in slight prolongation of the life of mice.[14] This prolongation was accentuated if sulfadiazine and adrenocortical extracts were used and their effectiveness was compared with sulfadiazine alone. On the other hand, unsuccessful attempts were made to increase the immunity of actively immunized mice by the use of adrenocortical extracts.[15]

The data presented in this paper show a slightly greater tendency for survival in ACTH-treated mice than in control mice, whereas mice treated with cortisone died more rapidly than controls. The discrepancies between these observations and those made using adrenocortical extracts remain unexplained. Possibly the effects of steroids other than cortisone present in cortical extracts may account for the difference. By the same token, ACTH would stimulate the adrenal cortex to produce additional steroids besides cortisone and hence its effects might be somewhat different from those produced by cortisone. The latter is closely related physiologically and chemically to compound F (17-hydroxycorticosterone) but is produced by the adrenal cortex only in small amounts or not at all.[16]

The wide variety of physiologic effects which follow the administration of ACTH and cortisone makes it difficult to determine any simple relationships between the hormones and resistance to infection. Such metabolic changes as the marked loss of nitrogen, the occasional development of diabetes, or other effects could conceivably so increase susceptibility to infection as to overbalance any enhancement of resistance induced by some different actions of the hormones.

The divergent action of ACTH and cortisone with respect to acute infection is difficult to assess. ACTH or cortisone may induce defervescence with relief of pain and clearing of evidence of toxemia,[1] yet the same hormones may inhibit the development of the inflammatory response[17] and thus depress resistance in some instances. Rabbits which are ordinarily resistant to infection with Group A streptococci may develop cardiac and renal lesions and succumb to such infections if they are receiving cortisone.[18] Similarly, tuberculous animals receiving cortisone have an altered inflammatory response as compared with control infected animals and may have more severe and more rapidly progressive lesions than their controls.[19, 20] Rapid progression of tuberculous lesions has occurred in some patients subsequent to the administration of ACTH or cortisone, despite the diminution of fever, increased appetite and sense of well-being which was observed during the period of administration of the hormones.[21, 22]

It is apparent that ACTH and cortisone are not to be considered as adjuncts to the therapy of infectious diseases, at this time. Their use in these circumstances should be considered experimental and should be undertaken only under carefully controlled conditions and with great caution. Certain of the effects of the hormones, however, require further study, in order to evaluate both their relationship to immune processes and the significance of some of the differences in the responses that have been observed in patients and in experimental animals.

SUMMARY AND CONCLUSIONS

Mice receiving ACTH or cortisone in doses adequate to maintain eosinopenia for six days lose 13 to 25 per cent of their body weights in that period of time.

The mortality rate of mice infected with virulent pneumococci is not decreased by treatment with ACTH or cortisone. Mice receiving ACTH tend to live a few hours longer than controls. Mice receiving cortisone usually die sooner and have a slightly higher mortality rate than controls.

Mice partially protected with specific antiserum are not afforded additional protection by the use of ACTH or cortisone. Again, animals receiving cortisone die more rapidly than control animals.

Mice infected with influenza virus are not protected by the hormones, and the rate of multiplication of the virus in the infected mouse lung is not altered by treatment with ACTH.

The divergent action of the adrenal hormones in inducing marked improvement in the clinical symptomatology of the patient with some acute infections but in not benefiting, or occasionally aggravating, infections in experimental animals makes it inadvisable to use ACTH or cortisone as general therapeutic agents in clinical infections until their role in such diseases is more clearly defined.

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EFFECT OF OXYGEN TENSION AND OF pH ON THE SICKLING AND MECHANICAL FRAGILITY OF ERYTHROCYTES FROM PATIENTS WITH SICKLE CELL ANEMIA AND THE SICKLE CELL TRAIT

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DURING the past few years, several types of study have defined important differences between sickle cell anemia and the sickle cell trait. Two groups of workers have found that the post-transfusion survival time of trait erythrocytes is normal while that of sickle cell anemia red cells is greatly shortened.[1, 2] London, Shemin, and Rittenberg have observed an in vitro synthesis of heme from tagged glycine by blood from patients with sickle cell anemia, but were unable to demonstrate this phenomenon when blood of individuals with the sickling trait was used.[3] Pauling and associates[4] have discovered that the hemoglobin in sickle cell anemia has a different electrophoretic mobility from normal hemoglobin and have postulated that there is a difference in the number or kind of ionizable groups in the two hemoglobins. Subjects with the trait were found to have a combination of the two forms.[4, 5] Schneider and Levin have reported that erythrocytes from patients with sickle cell anemia, when injected into rabbits, produce antisera which agglutinate the red cells of individuals with sickle cell anemia, but not those of subjects with the trait.[6] On the basis of genetic studies, Neel has proposed that patients with sickle cell anemia are homozygous with respect to the sickle cell gene, while those with the trait carry a heterozygous gene combination.[7]

None of these investigations, however, actually explains the following enigma. In atmospheres of carbon dioxide or in the presence of reducing agents,[8, 9, 10] nearly 100 per cent of both trait and anemia erythrocytes become sickled. The capacity to sickle is possessed equally, therefore, by the two types of red cells. Yet their in vivo behavior is so different that a shortened survival time and hemolytic anemia are characteristic of sickle cell anemia, while a normal survival time and normal total red cell values are found in the sickle cell trait. A clue to the explanation of this difference is provided: (a) by the demonstration that when erythrocytes are sickled, they are less resistant to mechanical trauma,[11, 12, 13] and (b) by the observation that cells from patients with sickle cell anemia sickle more readily than do trait erythrocytes. No satisfactory quantitative measurements of the difference in susceptibility to sickling have been made, but Sherman did show that sickling occurs at a higher oxygen tension in sickle cell anemia than in sickle cell trait.[14] He regulated the oxygen

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tension, however, by producing various degrees of a partial vacuum. In addition, Hahn and Gillespie observed that a decrease in hydrogen-ion concentration increases the rate of sickling and that anemia cells are more susceptible to these changes than are trait erythrocytes.[15]

The investigations to be described in the present report were designed to determine with greater precision (1) the levels of oxygen tension at which sickling occurs in red cells from patients with both types of sickle cell disease. (2) the effect of sickling on mechanical fragility, and (3) the effect of pH on sickling. It was found that the mechanical fragility of red cells paralleled the degree of sickling in all experiments. Lowering the pH of the suspension of cells was found to increase the degree of sickling at higher oxygen tensions. The erythrocytes from patients with sickle cell anemia tended to assume abnormal shapes at higher oxygen concentrations than did trait cells. In general, the oxygen tension had to be reduced to a partial pressure of 15 mm. Hg or less before appreciable sickling of trait cells occurred. Since the oxygen tension of tissues is seldom reduced to that level or lower,[16] and since sickling of anemia erythrocytes did occur at oxygen tensions likely to be found in tissues or in venous blood, it is suggested that this quantitative difference in the ease with which sickling occurs may account, at least in part, for the different clinical and hematologic manifestations of sickle cell anemia and the sickle cell trait.

METHODS AND EXPERIMENTAL CONDITIONS

Blood obtained by venipuncture and oxalated with the standard potassium ammonium oxalate mixture[17] was adjusted to a hematocrit of 40. One-half milliliter was then placed along with ten glass beads in a 50 ml. Erlenmeyer flask and rotated at 32 revolutions per minute on a wheel capable of holding four such flasks. The distance from the center of the wheel to the center of each flask bottom was 4.5 centimeters. The flasks were fitted with two-holed rubber stoppers. Through one hole was inserted an inlet tube which reached within 1 cm. of the bottom of the flask. Through this tube, a steady stream of an appropriate, hydrated gas mixture was permitted to flow at atmospheric pressure during the time the flasks were rotated. The second hole in the stopper served as the outlet. The mechanical difficulty of passing the gas mixture through the flasks while they were being rotated on the wheel was solved by use of a pressure rotating valve. The same gas mixture had to flow through all four flasks being rotated at any one time; by employing two wheels, it was possible to use two gas mixtures and to do two experiments simultaneously.

In order to make certain that the concentrations of oxygen and of carbon dioxide were the same for all experiments, gas mixtures were prepared in large cylinders of 2,200 cu. ft. capacity as follows:

MIXTURES WITHOUT CO_2		MIXTURES WITH CO_2		
O_2	N_2	O_2	N_2	CO_2
11.9%	88.1%	11.5%	78.5%	10%
7.7	92.3	7.8	82.2	10
5.8	94.2	5.8	84.2	10
4.0	96.0	4.1	85.9	10
2.1	97.9	1.9	88.1	10
0	100.0	0	90.0	10

These mixtures were prepared by weight by the Linde Oxygen Company. Samples collected at random were analyzed for O_2 and CO_2 by the method of Van Slyke and Sendroy[18]; in each instance the results agreed with the foregoing analysis within the limits of error of

the analytic method.* Hydration of the gases was accomplished by bubbling them through two water bottles (containing fritted gas dispersers and glass wool to break up the bubbles) placed between the tank and the rotating valve. The flow of gas was kept constant at approximately 1,200 ml. per minute for all four flasks on a wheel, or about 300 ml. per minute per flask. All experiments were done at room temperature (23.5° to 31.0° C.).

With the few exceptions noted later, the period of rotation and of exposure of the blood to any given gas mixture was standardized at 90 minutes. At the end of this time, one of the flasks was used for the determination of pH, one for estimating the per cent of sickling, and two for determination of the mechanical fragility. The sample for pH measurement was diluted with 3 ml. of 0.85 per cent saline and then aspirated into an airtight hypodermic glass electrode while the gas was still flowing through the flask. The pH was read immediately at room temperature with a Beckman hydrogen-ion meter. Into the second flask, also while the gas was still flowing, 2 ml. of 10 per cent Formalin in 0.85 per cent saline were injected. Four minutes were allowed for fixing the red cells; moist cover slip preparations were made and then sealed with petrolatum. The number of sickled and abnormal forms per 1,000 cells was counted. This method constitutes an adaptation of the technique described by Sherman.[14] In the final two flasks, mechanical fragilities were determined in duplicate by the method of Shen[11]; oxyhemoglobin was measured in an Evelyn photoelectric colorimeter.

In some of the experiments, the hydrogen-ion concentration was altered by buffers. Details of these special conditions will be given when the experiments are described.

All of the patients with sickle cell anemia, with one exception, had the characteristic clinical manifestations of the disease: an anemia, reticulocytosis, elevated icteric index, more than 30 per cent abnormal forms with Sherman's test; and all have required repeated transfusions for support. No transfusions, however, had been given within four months prior to these studies with the exception of two patients studied for the effect of pH on sickling. The one patient with sickle cell anemia who did not show the usual clinical manifestations was Patient J. B., an adolescent boy, 16 years of age. His red blood cell count has varied from 3.4 to 4.35 million per cubic millimeter. Hemoglobin values have varied from 11.1 to 12.2 Gm. per cent, and the packed cell volumes from 32 to 37 per cent. The reticulocytes have ranged from 3.4 to 11.3 per cent. The Sherman test on three occasions showed from 14.7 to 15.7 per cent abnormal forms. The icterus index on two occasions was 15. The direct reading bilirubin was 0.37 mg. per 100 ml. of plasma and the indirect bilirubin equaled 1.19 mg. per 100 ml. of plasma. Urine urobilinogen was within normal limits but the fecal urobilinogen was elevated to 1,088 Ehrlich units per 100 Gm. of feces. The Coomb's test was negative. The patient has not had any of the clinical manifestations of sickle cell anemia except for delayed puberty and subnormal growth. No transfusions have been required. The subjects with sickle cell trait had no clinical or laboratory evidences of sickle cell anemia, although several were anemic as a result of chronic hemorrhage.

RESULTS

A. *Mechanical Fragility, pH, and Degree of Sickling After Exposure to Low Oxygen tension in O_2-N_2 Mixtures.*—The first experiments were done in atmospheres of O_2 and N_2 alone to determine the difference in behavior of sickle cell anemia and trait erythrocytes. Determinations were made on the blood from six patients with sickle cell anemia and from five subjects with the sickle cell trait. Results are tabulated in Table I. The extremes of variation, outlining the range of values, are graphically illustrated in Fig. 1. In this and subsequent figures, the per cent of hemolysis is graphed as the expression of mechanical fragility, and the approximate oxygen tensions given by the different oxygen concentrations in the gas mixtures are indicated. Analysis of the data shows:

*The authors are indebted to Mr. C. E. Rhein of the Linde Oxygen Co. for making arrangements to have these special tanks prepared, and to Dr. John C. Tinsley for the manometric analyses.

TABLE I. HEMATOLOGIC DATA OF SICKLE CELL EXPOSED TO DIFFERENT OXYGEN CONCENTRATIONS AND

PATIENT									11.9%			
NAME	SEX	AGE	DIAGNOSIS SICKLE CELL	HB. (GM./ 100 C.C.)	R.B.C. (MILLIONS/ MM.^{3})	RETICS (%)	S* (%)	MFW (%)	MF^x (%)	S^y (%)	pH	MF^x (%)
L. B.	F	33	Anemia	10.1	2.90	15.4	30.1	3.8	5.4	5.3	7.68	5.4
K. D.	F	39	Anemia	7.8	2.04	25.0	32.2	12.0	13.4	9.9	7.44	12.3
E. P.	M	19	Anemia	8.4	2.70	46.2	35.6	6.2	7.5	4.3	7.65	4.9
F. O.	M	20	Anemia	8.1	2.44	44.0	35.6	5.6	9.0	13.2	7.54	
T. J.	F	29	Anemia	6.0	1.71	21.5	34.7	6.0	5.7	2.1	7.62	5.4
J. B.	M	16	Anemia	11.6	3.90	5.4	15.2	5.5	4.8	1.2	7.62	5.1
A. F.	F	42	Trait	11.5	4.90	3.0	10.1	2.1	2.3	2.7	7.79	2.2
A. M.	F	29	Trait	13.7	4.36	3.3	3.3	3.5	3.5	1.5		4.0
J. McM.	F	12	Trait	12.5	4.60	1.6	2.6	3.7	3.1	1.7	7.68	2.5
G. H.^{z}	M	54	Trait	6.9	2.20	3.3	1.2	4.8	5.6	1.4	7.38	4.3
B. G.	F	29	Trait	10.1	4.44	8.4	1.4	3.9	2.9	0.5	7.30	2.5

*Abnormal and sickle cells. Sherman test.
†Concentration of oxygen. Remainder of gas mixture was nitrogen.
w, Mechanical fragility of cells in terms of per cent hemolysis.

FIGURE 1

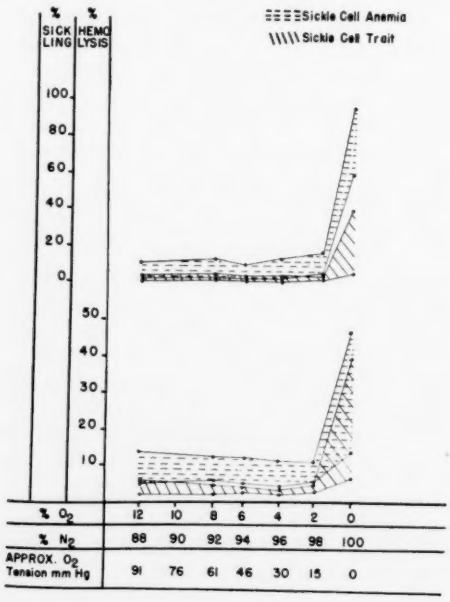

Fig. 1.—Effect of varying concentrations of oxygen and nitrogen on anemia and trait erythrocytes.

(1) that in an atmosphere of 2 per cent oxygen the number of sickled cells increased in four of six specimens from sickle cell anemia but not with trait erythrocytes; (2) that in pure nitrogen there was a definite increase of sickling in all specimens; (3) that at any given O_2 tension the degree of sickling for anemia cells was greater than for trait erythrocytes; and (4) that the changes in mechnical fargility of both types of cells paralleled closely the increase in the number of sickled forms.

ANEMIA AND SICKLE CELL TRAIT RED BLOOD CELLS AS PROVIDED BY GAS MIXTURES CONTAINING OXYGEN NITROGEN

OXYGEN CONCENTRATION†													
7.7%			5.8%			4.0%			2.1%			0%		
MFx (%)	Sy (%)	pH	MFx (%)	Sy (%)	pH	MFx (%)	Sy (%)	pH	MFx (%)	Sy (%)	pH	MFx (%)	Sy (%)	pH
5.4	5.6	8.04	6.1	5.3	7.63	4.0	4.5	7.79	4.8	4.9	7.75	13.5	59.2	7.72
12.3	12.3	7.65	12.1	9.2	7.65	11.8	9.5	7.91	11.8	11.1	7.72	47.0	89.4	7.81
4.9	6.3	7.90	8.0	4.2	7.83	7.5	6.0	7.90	9.6	12.9	7.51	35.0	88.4	7.63
			6.3	7.2	7.49	6.2	13.1	7.46	5.5	16.7	7.59	34.2	91.2	7.46
5.4	2.3	7.54	5.7	4.4	7.48	6.8	5.0	7.58	7.1	14.2	7.68	31.4	96.5	7.41
5.1	1.5	7.63	4.5	1.7	7.49	4.0	1.8	7.52	6.1	15.9	7.75	28.8	91.0	7.76
2.2	3.4	7.50	2.4	2.1	7.64	3.1	3.8	7.63	2.7	3.3	7.70	17.9	82.0	7.73
4.0	1.5		3.6	1.6		3.8	1.9		3.5	2.6		9.0	39.4	
2.5	1.7	7.72	4.1	2.3	7.62	3.1	1.4	7.63	3.0	1.2	7.43	9.2	20.1	7.38
4.3	1.4	7.30	4.0	1.1	7.48	3.7	1.1	7.31	4.8	1.2	7.43	8.0	6.6	7.45
2.5	0.6	7.32	1.9	0.5	7.38	2.9	0.5	7.19	2.9	3.9	7.20	6.7	11.8	7.19

x, Mechanical fragility after exposure to gas mixture.
y, Percentage of sickle cells following exposure to gas mixture,
z, Patient had anemia associated with nitrogen retention.

These results were disappointing in several respects. The difference in behavior of anemia and trait cells was not as great as had been expected. Furthermore, even though 30 per cent or more of the red cells are usually sickled in the venous blood of patients with sickle cell anemia where the oxygen tension is normally 30 mm. Hg or more, it was necessary to reduce the oxygen tension to less than half that level to obtain comparable degrees of sickling. A probable explanation for this result was provided by the pH values which tended to be higher than normal. It seemed likely that the constant flow of gas through the flasks washed away CO_2 and caused the blood to become more alkaline. In order the compensate for this effect, the experiments were repeated with gas mixtures that contained approximately the same concentration of O_2 but had 10 per cent CO_2 added.

B. *Mechanical Fragility, PH, and Degree of Sickling After Exposure to Low Oxygen Tension in O_2-N_2 Mixtures Containing 10 per cent CO_2.*—Blood from the same subjects was used. Results are recorded in Table II; the range of values is indicated in Fig. 2. The addition of 10 per cent CO_2 to the gas mixtures lowered the pH of blood samples to between 6.63 and 7.08, and led to sickling of both types of cells in higher concentrations of oxygen. In four of the six specimens from patients with sickle cell anemia, sickling began to increase at an O_2 concentration of 5.8 per cent; in the other two instances, the increase occurred at 4.1 per cent O_2. By contrast, in blood from subjects with the trait, a higher percentage of sickled forms did not appear until the O_2 concentration had been lowered to 1.9 per cent in four instances and to 4.1 per cent in the remaining case. As in the previous experiments, mechanical fragility increased in proportion to the degree of sickling. The average values for the percentage of sickled cells at each oxygen concentration show that sickle cell anemia erythrocytes tend to assume their abnormal shape at a distinctly higher oxygen tension than do trait cells. There is, however, a tendency for some overlapping to occur and the curves (Fig. 2) which describe the extremes of the results obtained are not widely separated.

TABLE II. HEMATOLOGIC DATA OF SICKLE CELL ANEMIA AND SICKLE CELL TRAIT RED BLOOD CELLS EXPOSED TO DIFFERENT OXYGEN CONCENTRATIONS AS PROVIDED BY GAS MIXTURES CONTAINING OXYGEN, NITROGEN, AND CARBON DIOXIDE†

PATIENT			OXYGEN CONCENTRATION*																	
			11.5%			7.8%			5.8%			4.1%			1.9%			0%		
NAME	DIAGNOSIS SICKLE CELL	MF^w (%)	MF^x (%)	S^y (%)	pH	MF^x (%)	S^y (%)	pH	MF^x (%)	S^y (%)	pH	MF^x (%)	S^y (%)	pH	MF^x (%)	S^y (%)	pH	MF^x (%)	S^y (%)	pH
L. B.	Anemia	3.8	4.8	3.3	6.91	4.6	4.2	6.84	10.7	16.5	6.92	34.3	88.9	6.92	43.2	96.5	6.93	37.2	96.7	6.83
K. D.	Anemia	12.0	11.2	8.5	6.99	8.0	8.9	6.98	12.8	11.1	6.92	16.3	14.4	6.98	40.3	89.2	6.88	45.0	93.2	6.63
E. P.	Anemia	6.2	5.5	5.5		6.2	12.7		7.8	6.5		18.2	35.9		36.5	96.5		33.5	96.7	
F. O.	Anemia	5.6	6.2	9.6	6.97	11.5	8.4	6.96	13.0	16.2	6.88	48.8	95.2	6.81	40.7	97.6	6.97	47.6	98.5	6.82
T. J.	Anemia	6.0	6.3	5.9	6.82	7.1	8.8	6.98	12.2	7.6	6.88	18.8	29.9	6.95	36.0	98.9	6.89	36.5	99.3	6.87
J. B.	Anemia	5.5	4.5	1.7	6.82	5.5	1.6	7.01	12.3	12.5	7.08	18.2	57.0	7.00	32.4	94.2	6.95	42.3	94.9	6.99
A. F.	Trait	2.1	2.8	2.3	6.79	4.1	3.2	6.90	3.0	2.7	6.91	9.0	16.5	6.75	28.0	92.7	6.77	32.2	94.9	6.83
A. M.	Trait	3.5	5.0	1.3		4.5	0.9		4.7	0.7		4.9	1.0		13.6	14.5		31.3	79.0	
J. McM.	Trait	3.7	4.5	0.9	6.92	3.5	1.0	6.92	3.8	1.5	6.89	4.5	1.3	6.93	11.8	25.6	6.79	28.5	61.1	6.75
G. H.	Trait	4.8	5.3	1.0	6.86	5.0	1.1	6.75	4.7	1.8	6.89	5.7	2.5	6.91	8.5	8.9	6.87	37.8	81.9	6.79
B. G.	Trait	3.9	3.6	2.0	7.00	3.1	0.5	7.04	4.8	1.0	6.98	4.0	2.0	7.00	15.5	19.2	7.04	24.6	35.6	6.99

*Concentration of oxygen. Remainder of gas mixture was 10 per cent carbon dioxide and nitrogen.

†For other hematologic data see Table I.

w, Mechanical fragility of cells.

x, Mechanical fragility after exposure to gas mixtures.

y, Percentage of sickle cells following exposure to gas mixtures.

Figs. 3 and 4 contrast for sickle cell anemia and sickle cell trait, respectively, the data obtained with the two types of gas mixtures. They emphasize the fact that with CO_2 added, sickling was produced at higher oxygen concentrations. Since this effect could have been produced either by the CO_2 itself or by the lowered pH, the following experiments were done in an attempt to determine which factor was responsible for the change.

FIGURE II

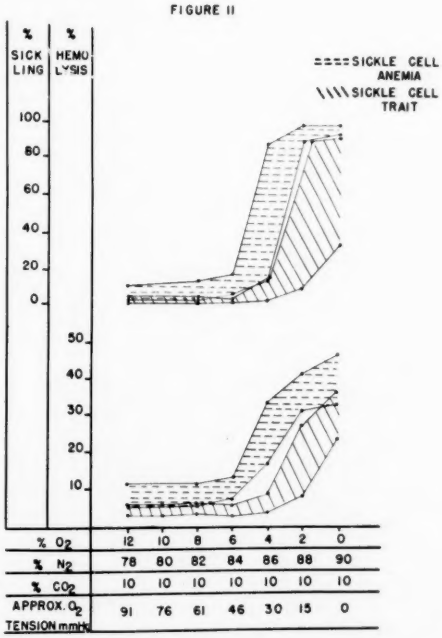

Fig. 2.—Effect of varying concentrations of oxygen and nitrogen with 10 per cent CO_2 on anemia and trait erythrocytes.

C. *Effect of pH on Sickling and on Mechanical Fragility.*—In order to test the effect of hydrogen-ion concentration on the susceptibility of erythrocytes to sickling, three types of experiments were done: (1) the pH of blood exposed to the O_2-N_2 gas mixtures was lowered by addition of a buffer to values approximating those found when the gas mixtures contained CO_2; (2) the pH of blood exposed to the O_2-N_2-CO_2 gas mixtures was raised by addition of a buffer to values approximating those found when the gas mixtures contained only O_2 and N_2; (3) the pH of blood exposed to 2.1 per cent O_2 and 97.9 per cent N_2 was varied by the use of buffers through a range of 6.5 to 7.8. The results demonstrate that it is pH rather than the presence or absence of CO_2 in the gas mixtures which influences the susceptibility of cells to sickling at low oxygen tensions.

(1) *Effect of Phosphate Buffer of pH 6.3 on the Sickling and Mechanical Fragility of Erythrocytes Exposed to O_2-N_2 Gas Mixtures:* Isotonic phosphate buffers were prepared by mixing appropriate amounts of 0.1 molar aqueous solutions of K_2HPO_4 and NaH_2PO_4. In exploratory experiments it was determined

that such a phosphate buffer of pH 6.3 would control the pH of the blood sample so that at the end of 90 minutes' exposure to O_2-N_2 mixtures during rotation in the Erlenmeyer flasks, the blood would have a pH of approximately 6.9. The actual values observed varied from 6.72 to 6.95 and corresponded well with the pH values obtained for blood exposed to gas mixtures containing 10 per cent CO_2 (Table II). Addition of buffer was made in the following manner. Each blood sample was first adjusted to a hematocrit of 40. Plasma equal to 50 per cent of the total adjusted volume was then removed and replaced by the 6.3 phosphate buffer.

FIGURE III

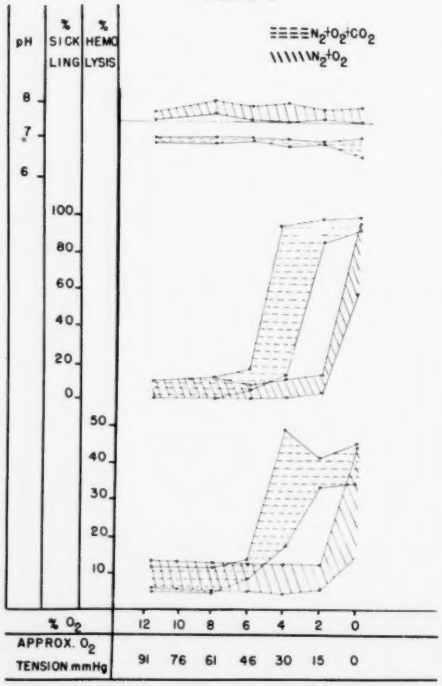

FIGURE IV

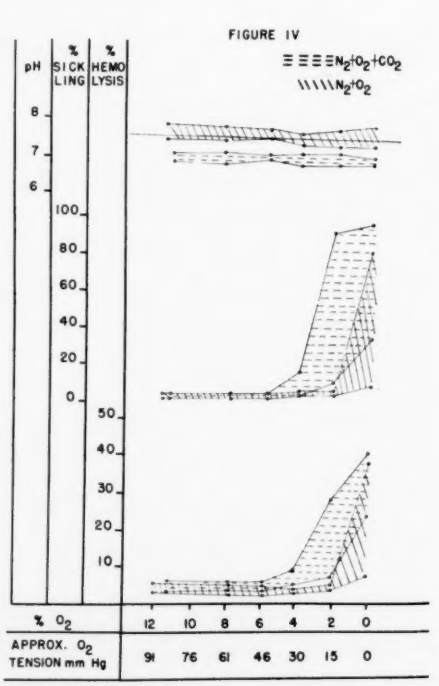

Fig. 3.—Effect of the two gas mixtures on anemia erythrocytes.
Fig. 4.—Effect of the two gas mixtures on trait erythrocytes.

Observations were made on the blood from two patients with sickle cell anemia and two subjects with the trait. Data for one of the patients with each type of sickle cell disease are charted in Figs. 5 and 6. The other two subjects showed similar results. In each instance increased sickling occurred at a higher oxygen concentration than when no buffer had been used and the pH, consequently, was higher. As in the previous experiments, mechanical fragility increased as sickling became more pronounced.

(2) *Effect of Sodium Bicarbonate on the Sickling and Mechanical Fragility of Erythrocytes exposed to O_2-N_2-CO_2 Gas Mixtures:* In order to raise the pH of blood samples exposed to the gas mixtures containing 10 per cent CO_2 to

approximately 7.4, it was necessary to use a 0.15 molar solution of sodium bicarbonate. Unfortunately, this solution caused partial hemolysis of the red cells unless it also contained 0.5 per cent sodium chloride. The final buffer used, then, was slightly hypertonic. Addition to the blood was made in the same manner as with the phosphate buffer.

FIGURE V

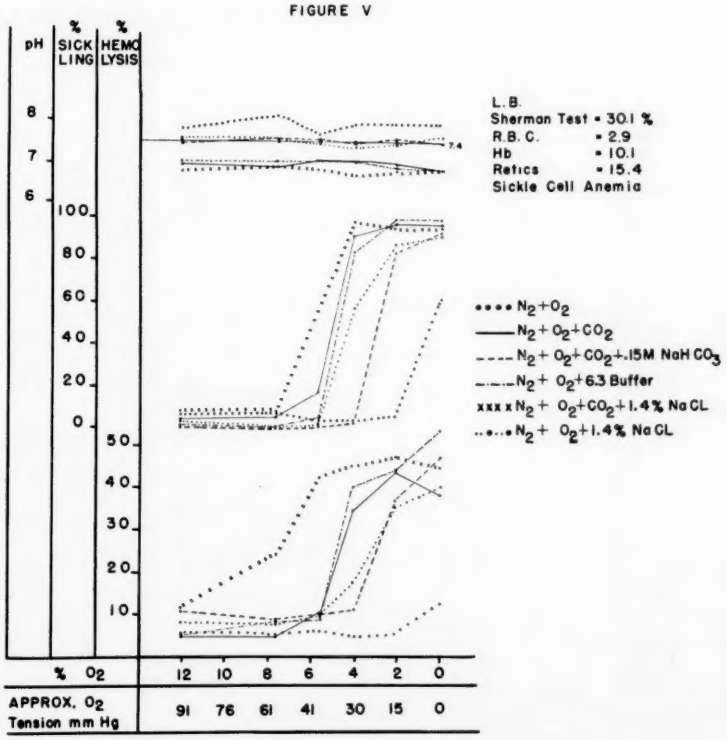

Fig. 5.—Effect of oxygen tension and hydrogen-ion concentration on anemia erythrocytes.

Observations were made on the blood of the same four subjects used in the experiments described in the previous section. Blood was exposed for 90 minutes of rotation to the gas mixtures containing 10 per cent CO_2. Results are charted for two patients in Figs. 5 and 6. The final pH values varied from 7.32 to 7.53; they were, therefore, approximately the same as those obtained with O_2-N_2 alone (Table I). The higher pH levels made both trait and sickle cell anema erythrocytes less susceptible to the sickling effects of low oxygen concentrations. Again, mechanical fragility of the cells paralleled the degree of sickling.

Because the sodium bicarbonate buffer was hypertonic, it was necessary to control the results by adding a sodium chloride solution of comparable hypertonicity, 1.4 per cent, in place of the buffer. This experiment was done on the blood from Patient L. B., with sickle cell anemia. With both types of gas mix-

tures (with and without 10 per cent CO_2), the cells sickled at higher concentrations of oxygen. The hypertonic sodium chloride, therefore, increased the susceptibilitiy of the cells to the sickling effect of low oxygen tensions (Fig. 5). This change is in the reverse direction from the effect of the bicarbonate buffer, and suggests that if it had been possible to use an isotonic bicarbonate buffer in these experiments, the higher pH might have been even more effective in decreasing the sickling and mechanical fragility at low oxygen concentrations.

FIGURE VI

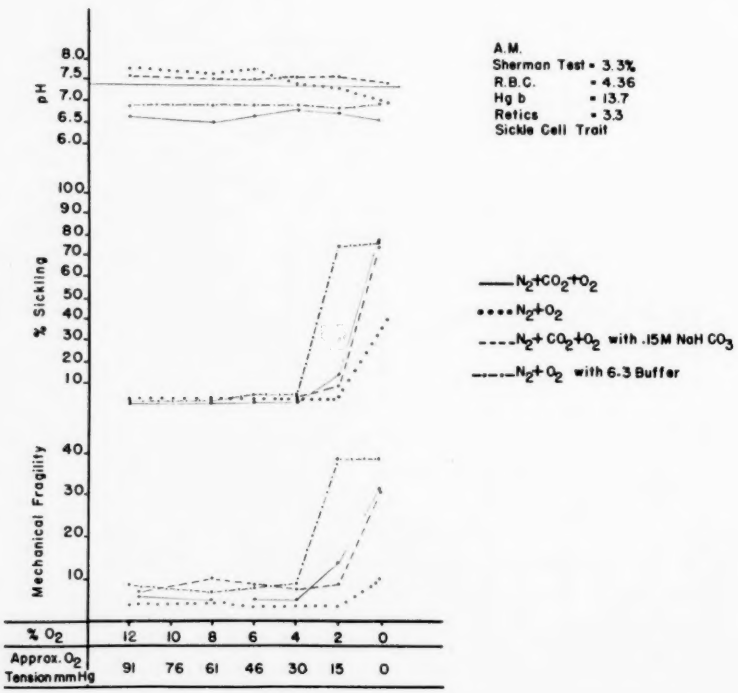

Fig. 6.—Effect of oxygen tension and hydrogen-ion concentration on trait erythrocytes.

(3) *Effect of pH on the Sickling Produced by 2.1 Per cent O_2 and 97.9 Per Cent N_2:* In order to study the effect of pH on the sickling, blood was exposed to 2.1 per cent oxygen and 97.9 per cent nitrogen under conditions similar to those used for all the previous experiments except that the time of rotation and exposure to the gas was reduced to 15 minutes. This gas mixture was selected because the oxygen concentration was low, yet by itself it did not cause marked sickling. The blood samples from five patients with sickle cell anemia and from five with the trait were studied. Phosphate buffers were added to give final pH values of from 6.5 to 7.8. Results are illustrated in Fig. 7. With pH values of 7.1 to 7.45, 50 per cent or more of the red cells from patients with sickle cell anemia became abnormal. Comparable degrees of sickling, however, were not produced in trait erythrocytes until the pH was lowered to 6.75

to 7.1. With this pH effect, therefore, it was also possible to demonstrate a difference in behavior of sickle cell anemia and trait erythrocytes, but here, too, some overlapping of results occurred.

FIGURE VII

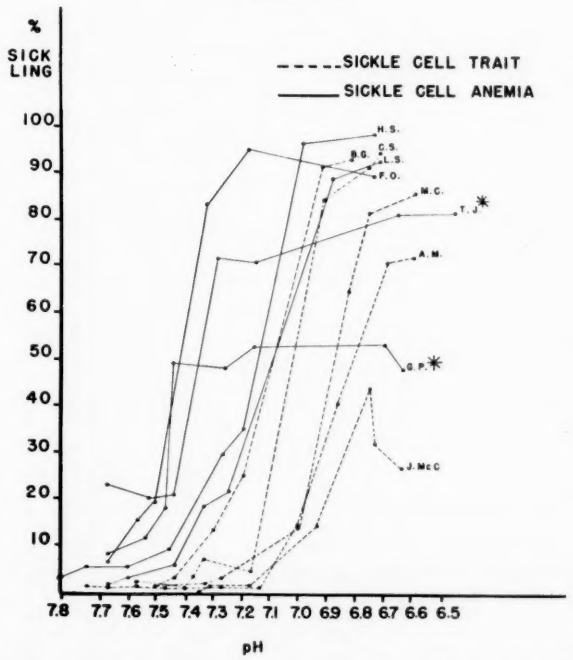

Fig. 7.—Effect of pH on anemia and trait erythrocytes. *Patients had been transfused prior to study.

DISCUSSION

Under the conditions of these experiments, the two factors which influenced susceptibility to sickling were the oxygen tension and the hydrogen-ion concentration. Decreases in either or both of these factors increased the degree of sickling. It would seem that the CO_2 tension is important only in so far as it influences the pH of the blood. The importance of oxygen tension and of pH in bringing about sickling has been noted previously,[14, 15] but the technique used in these earlier studies were often less precise and did not differentiate well the relative importance of the two influences. Other investigators have observed that reducing substances increase sickling.[8, 9, 10] Since oxidation reduction potentials were not measured in the experiments described in this report, no evaluation of the possible reducing effects of low oxygen tensions or of lowered pH can be made.

It has frequently been reported[14, 15, 19] that at an oxygen tension of 45 mm. Hg most of the erythrocytes of sickle cell anemia patients would assume the sickled form. In these investigations, however, an approximate oxygen tension of 30 mm. Hg was needed to produce an increase in the degree of sickling when

carbon dioxide gas mixtures were used. When the nitrogen-oxygen gas mixtures were used, an oxygen tension of zero was needed to produce a marked increase of sickling of the erythrocytes from sickle cell anemia patients.

The results of these experiments demonstrate that erythrocytes from patients with both sickle cell anemia and sickle cell trait withstand mechanical trauma normally when in the form of biconcave discs, but have an increased mechanical fragility when in the sickled state. Similar observations have been made by Shen, Castle, and Fleming,[11] by Diggs and Bibb,[13] and by Nickel.[12]

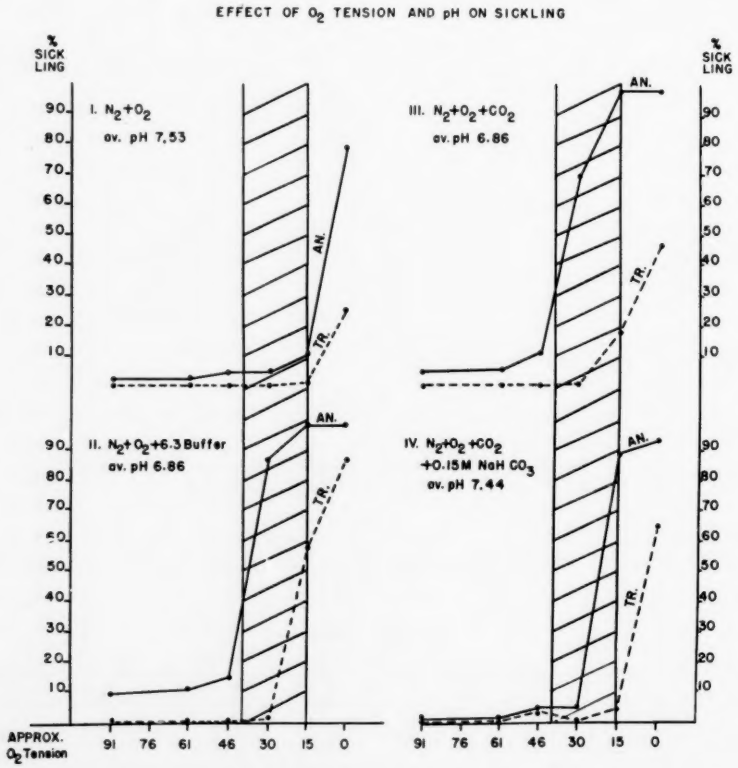

Fig. 8.—*AN.*, Average result of two patients with anemia. *TR.*, Average result of two patients with trait.

The principal reason this study was undertaken was to discover, if possible, any physiologic difference in the behavior of anemia and trait cells which would account for the accelerated hemolysis in sickle cell anemia and the normal erythrocyte values characteristic of sickle cell trait. The oxygen tension in tissues probably varies between 15 and 42 mm. Hg; with severe vasoconstriction it may apparently fall to 10 mm. Hg.[16] If one could demonstrate that sickle cell anemia erythrocytes would tend to sickle at oxygen tensions and hydrogen-ion concentrations regularly found in tissues while trait cells failed to do so, then one would have an explanation for the different manifestations of the two forms of sickle cell disease. In Fig. 8 the average data of two anemia and two

trait subjects are charted in an attempt to demonstrate such a difference. The vertical striated columns delineate roughly the oxygen tensions which might be found in the tissues. It is evident that the sickling of trait cells tends to be minimal at these levels whereas sickling of anemia cells does occur. If one plotted average values for all experiments, similar differences would be observed. However, the explanation is not too satisfactory because overlapping of results occurred and a sharp differentiation between cells from anemia and the trait could not always be made with these experimental techniques alone. This overlapping may occur in part because the red cells from patients with sickle cell trait may differ in their susceptibility or resistance to sickling. Such reasoning is in agreement with the recent observations of Wells and Itano who found the percentage of abnormal hemoglobin in sicklemia to vary from 24 to 43 per cent.[5] It is possible that a sharper difference in the behavior of the two types of erythrocytes might have been demonstrated had we been able to duplicate physiologic conditions more satisfactorily.

SUMMARY AND CONCLUSIONS

The erythrocytes of sickle cell anemia and sickle cell trait patients were studied by measuring the mechanical fragility and degree of sickling under different oxygen and hydrogen-ion concentrations.

The results demonstrate: (1) that the hemolysis produced by mechanical trauma of both types of red cells increased as the percentage of sickling increased; (2) that both pH and oxygen tension are important factors in regulating the degree of sickling; (3) that CO_2 produces its effect on sickling by lowering the pH; (4) that an oxygen tension of below 45 mm. Hg is needed to produce sickling of the erythrocytes from some sickle cell anemia patients; (5) that, in general, trait erythrocytes are more resistant to changes in both pH and oxygen tension than are anemia cells.

It is suggested that this difference in the susceptibility of sickle cell anemia and trait erythrocytes to sickling, plus the accompanying differences in mechanical fragility, may explain why sickle cell anemia and sickle cell trait differ in their clinical and hematologic manifestations.

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LABORATORY METHODS

EVALUATION OF THE MOUSE TEST FOR THE LABORATORY DIAGNOSIS OF TUBERCULOSIS

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IN A preliminary report[1] we found that gastric mucin significantly enhances the virulence of tubercle bacilli for pigmented strains of mice (line 1 dba, C3H and C57 black). Grossly visible lesions containing large numbers of tubercle bacilli were usually present ten days after intraperitoneal inoculation with the dosages and strains of tubercle bacilli studied. Several isolations of tubercle bacilli from patients were made ten to fifteen days after inoculation by use of line 1 dba or C3H mice and mucin. We also found that the number of isolations from patients could be significantly increased by feeding pigmented mice inoculated with mucin the cornmeal diet of Dubos and Pierce.[2]

An evaluation of the suitability of the mouse test for the routine laboratory diagnosis of tuberculosis was attempted in the present study. This was done by inoculating specimens from patients into mice, guinea pigs, and culture media simultaneously. A microscopic examination of the direct smear of each specimen was also done.

MATERIALS AND METHODS

Mice.—Line 1 dba mice weighing 13 to 15 grams, obtained from the Roscoe B. Jackson Memorial Laboratory, were used in all tests. Usually three or four mice were inoculated with each specimen, and the mice were kept in individual glass jars with metal tops. The mice were fed a diet composed of Purina dog Checkers and water until the time of inoculation, when the cornmeal diet of Dubos and Pierce[2] was substituted.

Mucin.—A 5 per cent suspension of granular mucin (Type 1701-W, Wilson Laboratories, Chicago) was prepared by suspending 5 Gm. in 100 c.c. of distilled water. The suspension was next filtered through gauze, adjusted to pH 7.4, and autoclaved. The prepared suspension was stored in the refrigerator at 3 to 5° C. until used.

Test Specimens.—Sputa, gastric lavage, etc., obtained from patients were prepared in the usual manner by the addition of 3 per cent sodium hydroxide and subsequent neutralization with sulfuric acid. Sputa were digested no longer than two hours and gastric lavage thirty minutes with sodium hydroxide. Following neutralization, the specimen was tested for sterility in thioglycollate media. If no growth occurred on the following day, the specimen was mixed with equal parts of 5 per cent mucin and inoculated subcutaneously (0.5 c.c.) into the back and intraperitoneally (0.5 c.c.) into each of three or four line 1 dba mice. The mice were sacrificed ten to twenty days after inoculation and impression

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smears were routinely made of all suspicious lesions. If no macroscopic lesions were present, organs such as the liver and spleen, the peritoneal fluid, and so on, were also smeared. All smears were fixed by heat and stained by Ziehl-Neelsen technique. The usual procedure was to kill one mouse of the group ten days after inoculation while the remainder were sacrificed at intervals up to twenty days after inoculation, depending on the initial findings.

A guinea pig and freshly prepared Petragnani's media were also inoculated simultaneously with each specimen. The guinea pigs were routinely sacrificed three months after inoculation and autopsied unless signs of infection were apparent sooner. Petragnani's media were incubated at 37° C. in stoppered tubes and observed daily until growth was first noted.

RESULTS

Two hundred and seventy-three specimens routinely collected from patients in Michael Reese Hospital for the laboratory diagnosis of tuberculosis were studied. The specimens were tested as received without selection. The types and number of specimens are shown in Table I. The number positive is based on combined results of smear, guinea pig, culture media, and mouse inoculations.

TABLE I. ROUTINE SPECIMENS FROM PATIENTS

SPECIMEN	NUMBER TESTED	NUMBER POSITIVE
Sputum	181	65
Gastric lavage	50	18
Urine	13	3
Spinal fluid	11	0
Bronchial lavage	6	1
Pleural fluid	6	0
Pus	5	3
Stool	1	0
Total	273	90

Table II shows the combined results obtained in comparative studies with the various specimens in direct smear, Petragnani's culture media, guinea pig, and mouse inoculations. Only 218 specimens of this series were inoculated into guinea pigs because of the shortage of these animals at the time this study was done. The direct smear consisted of examination of the concentrate as well as suspicious particulate matter, especially in sputum specimens. Petragnani's media were incubated for at least three months before being discarded.

TABLE II. COMPARATIVE RESULTS OF VARIOUS SPECIMENS TESTED IN DIRECT SMEAR, PETRAGNANI'S MEDIA, GUINEA PIGS, AND MICE

TEST METHOD	NUMBER OF SPECIMENS	NO. POSITIVE	PER CENT POSITIVE
Direct smear	273	57	20.9
Culture	273	90	33.3
Guinea pig	218	69	31.6
Mouse	273	67	24.5

The results obtained in Table II clearly indicate that the rapid mouse test in which the mice are sacrificed ten to twenty days after inoculation of the specimen plus mucin is inferior to Petragnani's media or guinea pig inocu-

lation. All of the specimens which were microscopically positive were also positive in mice. However, ten specimens which were microscopically negative were positive in the mouse test. Only two groups of mice of the sixty-seven positive failed to show macroscopic lesions. Both of these showed the presence of acid-fast bacilli in impression smears of the spleen, liver, or peritoneal fluid. Most of the positive mice showed tubercle-like macroscopic lesions ranging in size from 2 to 4 mm. at the site of both subcutaneous and intraperitoneal inoculation. About 5 per cent developed macroscopic lesions at the site of subcutaneous inoculation only and were negative intraperitoneally. On the other hand, none showed gross lesions intraperitoneally only. In every instance impression smears of gross lesions in positive mice stained by the Ziehl-Neelsen technique showed the presence of large numbers of acid-fast bacilli.

Most of the microscopically negative specimens which were positive in the culture or guinea pig were negative in the mouse. Approximately 95 per cent of the positive cultures showed growth in four to six weeks; 2 per cent were positive in three weeks and 3 per cent in six to eight weeks. Ninety-seven per cent of the guinea pigs were positive in six to eight weeks while 3 per cent were not positive until three months had elapsed.

DISCUSSION

No attempt was made to determine whether the mouse test would be as sensitive as culture media or guinea pig inoculation if longer intervals of time were allowed to elapse following inoculation. However, we have confirmed the observations of Patnode and co-workers[3] that mouse inoculation is of value in testing the pathogenicity of cultures with atypical growth in a relatively short time.

In the mouse test it is imperative that all gross lesions be smeared and examined microscopically for acid-fast bacilli because other organisms such as Actinomyces,[4] Nocardia, etc., may produce similar lesions when inoculated with mucin. Occasionally we have observed apparently typical lesions in which no acid-fast bacilli could be demonstrated. We regarded such lesions as being negative in tabulating results of the present study. It is not practical to inoculate mice intracerebrally for diagnostic work because of the high mortality due to the toxicity of the prepared specimens.[3] We observed no apparent toxicity in mice inoculated subcutaneously or intraperitoneally with the various specimens plus mucin in the present study.

The fact that the mouse fails to develop tuberculin sensitivity makes it impossible to detect spontaneous tuberculous infection in these animals. This disadvantage together with Glover's finding[5] that relatively few tubercle bacilli can produce recognizable tuberculous foci in mice by inhalation are objections which have been raised against using mice in diagnosis of tuberculosis. However, we feel that our positive findings in mice are valid because in most instances macroscopic lesions containing numerous acid-fast bacilli were present at the site of subcutaneous or intraperitoneal inoculation.

SUMMARY

Comparative studies were carried out of routine specimens from patients for the diagnosis of tuberculosis by the direct smear and simultaneous inoculation of Petragnani's media, guinea pigs, and line 1 dba mice. The various specimens inoculated into mice were previously mixed with gastric mucin and injected both subcutaneously and intraperitoneally. The mice were fed a cornmeal diet starting at the time of inoculation and were sacrificed ten to twenty days later. The results obtained indicate that the mouse is inferior to the guinea pig and culture media and cannot be recommended as a primary diagnostic procedure for the routine laboratory diagnosis of tuberculosis.

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A MICROMETHOD FOR PROTHROMBIN TIME DETERMINATIONS

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EXTENSIVE use of Dicumarol therapy has made frequent prothrombin time estimations imperative. Several methods of varying efficiency and complexity are available for the performance of this determination. Outstanding among these are the methods of Quick,[1] Smith, Warner, and Brinkhous,[2] the bedside test of Smith and associates,[3] the microprothrombin time of Kato,[4] and the plasma dilution method of Shapiro.[5] The more accepted and accurate of these methods require venipuncture, a procedure dreaded by patient, physician, and technician alike when repeated frequently over an extended period. A method was therefore sought which would have the accuracy of the methods requiring venipuncture and yet possess the ease of finger puncture. The micromethod described below uses the anticoagulant immediately for the dilution of Shapiro's method.[5]

MATERIALS AND METHODS

Reagents.—

1. One hundred and twenty-five ten thousandth (0.0125) molar sodium oxalate (0.168 per cent) is prepared in 0.9 per cent sodium chloride.
2. Difco or any other acceptable thromboplastin, prepared as directed in the accompanying literature.
3. Fortieth molar calcium chloride.

Procedure.—

Pipettes 0.2 ml. (to contain) are prepared by rinsing with the oxalate reagent described and allowing them to drain. Drying may be hastened by passing air through the pipettes. Samples are prepared by adding 0.2 ml. of blood, obtained by deep finger puncture with a lancet, to 0.77 ml. of the oxalate reagent described in a small (750 by 10 mm.) test tube. The blood is expelled and the pipette is rinsed several times with the mixture. The tube is stoppered and inverted at least ten times. This is followed by centrifugation and removal of the supernatant. The supernatant is then placed in a constant-temperature water bath at 37° C., as are the thromboplastin and calcium chloride reagents. After five minutes' incubation, 0.1 ml. of thromboplastin and 0.1 ml. of calcium chloride are pipetted into 75 by 100 mm. tubes. Then 0.1 ml. of the supernatant plasma from unknown and control, already at 37° C., are blown into the thromboplastin-calcium chloride mixtures. A stop watch is started at this moment and complete continuous mixing is maintained by passing a nichrome needle with a small loop through the mixture in even strokes. The end point is the time of appearance of strands of fibrin which are lifted above the liquid surface by the passing loops.

RESULTS AND DISCUSSION

This micromethod is a modification of Shapiro's diluted plasma prothrombin time. Shapiro[5] as well as Unger and Shapiro[6] has emphasized that the use of 12.5 per cent plasma is more sensitive for the detection of fine alterations in

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prothrombin concentration than the undiluted plasma, while retaining a clear-cut end point in cases of hypoprothrombinemia. As mentioned previously, the micromethod described incorporates the dilution factor with the anticoagulant, resulting in a diluted plasma specimen immediately at the time blood is drawn. A close correspondence between values obtained by Shapiro's macromethod and the micromethod has been observed. The end point was always detected with ease on patients receiving anticoagulant therapy and reflected the degree of hypoprothrombinemia as determined by the usual methods with and without dilution, with accuracy. (Table I.)

TABLE I. PROTHROMBIN TIMES ON RANDOM PLASMA SAMPLES

PLASMA SAMPLE	QUICK'S METHOD (SEC.)	SHAPIRO'S METHOD (SEC.)	MICROMETHOD (SEC.)
1	15	35	36
2	15	37	37
3	14	35	36
4	16	39	41
5	21	61	59
6	16	40	41
7	30	83	84
8	35	87	87
9	40	91	93

The advantage of the micromethod is its ease of performance. It provides relief from repeated venipunctures. It makes accurate prothrombin time determinations feasible in children and in adults with poor veins. The micromethod using capillary blood approximates more closely the actual blood coagulation as it occurs physiologically in the body, for most spontaneous bleeding is capillary in origin. Coagulation from the time of puncture is under complete control. The tourniquet effect, difficult to overcome when venous blood is used, is lost with the free natural flow of blood following deep finger puncture. The difficulty in entering the vein in many cases, particularly after repetitions of the puncture procedure, causes variation in the amount of tissue thromboplastin liberation, which affects the subsequent technical procedure, with no possibility of correction for this variable error. This great source of error in prothrombin time determinations which will vitiate a most accurate laboratory procedure is better controlled by a uniform type of finger puncture.

The micromethod also provides a suitable emergency bedside test since centrifugation may be omitted and the whole blood can be used and a thermos bottle can be substituted for the constant-temperature water bath.

CONCLUSION

A micromethod for prothrombin time determinations, which can be performed on capillary blood and is comparable in accuracy to results obtained by the methods of Shapiro and Quick, has been described. It can be adapted for emergency use.

The author gratefully acknowledges the technical assistance of Mrs. Anne Sachs and Gilbert S. Trelawny.

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AN INEXPENSIVE CARRIER AMPLIFIER FOR USE WITH RESISTANCE WIRE MANOMETERS

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INTRODUCTION

THE optical registration of intracardiac pressure pulses during the course of cardiac catheterization can be accomplished with reasonable fidelity and accuracy by making use of resistance wire manometers and photographically recording galvanometers.[1, 2, 3] In many applications the use of sufficiently long optical levers and sufficiently sensitive galvanometers makes possible the use of these instruments directly coupled without electronic amplification. In some instances, however, amplification of the output of the pressure transducer is desirable. It is the purpose of this paper to describe a simple, inexpensive carrier amplifier that was constructed for use with a Statham† P23A pressure transducer and a Heiland‡ type G galvanometer at 100 cm. optical distance.

A transducer of the type used in this application consists, essentially, of a normally balanced bridge circuit of resistive elements (Fig. 1) and a mechanical linkage system which causes an applied pressure to strain the elements of the bridge. Straining of the resistive bridge arms changes their resistances and causes the bridge to become unbalanced. The output voltage obtained from the bridge of the transducer under consideration is proportional to the presssure applied to the mechanical linkage system.

The transducer bridge may be excited either by means of a constant (D.C.) voltage or a sinusoidally varying (A.C.) voltage. When the transducer is excited by means of a D.C. voltage the amplification system must be capable of amplifying zero frequency (D.C.) voltages. In general, D.C. amplification systems are considerably more complex, cumbersome, and expensive than carrier systems. Moreover, such systems are subject to problems of drift resulting from thermal effects in the vacuum tubes used. Finally, D.C. amplification systems require the use of high performance regulated power supplies, providing three or more positive and negative voltages.

Carrier amplification systems, which are applicable when A.C. transducer excitation is used, are simple, inexpensive, and reliable. A complication, however, which arises from the use of such systems is that of "detecting," i.e.

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‡Heiland Research Corporation, 130 E. 5th Ave., Denver 9, Colo.

measuring the instantaneous amplitude of the A.C. voltage of the bridge output. Most of the common detecting systems exhibit nonlinear performance, the output of the detector being not directly proportional to the instantaneous amplitude of the A.C. input. The most straightforward method for eliminating this difficulty is to amplify the alternating voltage to a fairly large amplitude (20 or more volts) before detection. Detectors yielding almost perfectly linear performance at these amplitudes are commonly available. This is not possible in cases in which the signal input to the amplification system may occasionally go to zero as is the case in the type of measurement under consideration. Moreover, when the voltage amplitude at the signal source (the transducer) is very small, the amount of amplification required to increase it to a level sufficient for good linear detection may be extremely large. When this is the case, such effects as thermal "noise" and instability may make the realization of such a system impossible.

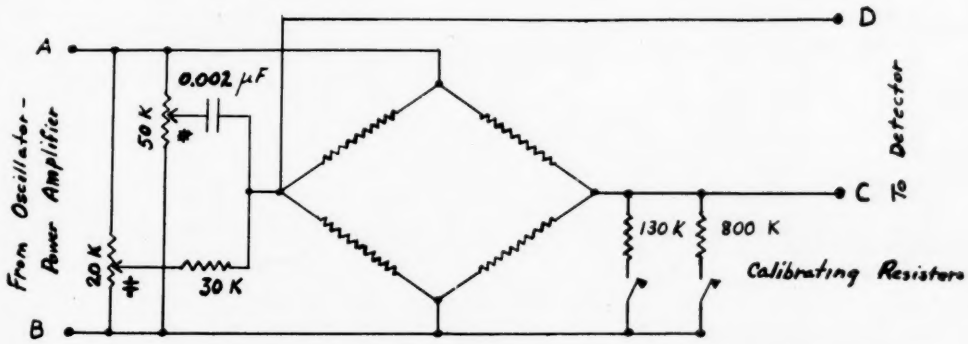

Fig. 1.—The bridge circuit. Included in this diagram are the bridge resistance (‡) balancing and the stray capacity (*) balancing circuits and the calibrating resistors (for discussion see text).

A system for obtaining excellent linear detection at low signal amplitudes will be described in the following section. The availability of such a detection system together with the inherent advantages of A.C. amplification systems, as contrasted to D.C. systems, has led to the selection of a carrier system for this application.

DESCRIPTION OF SYSTEM

The amplification system as used here is composed of two parts, an oscillator-power amplifier to supply the carrier excitation for the bridge contained in the pressure transducer and a carrier amplifier-detector for the purpose of amplifying and detecting the signal output of the pressure transducer. The power supply (not shown) for these includes a 300 volt nonregulated component and a 250 volt supply regulated by a conventional cascode regulator employing one 6Y6, one 6SL7, and one VR 105 vacuum tube.

To be satisfactory, the oscillator-power amplifier must supply a reasonably distortionless sinsoidal signal of constant amplitude (9 volts rms, in this instance) to the transducer bridge (250 ohms input impedence). The circuit shown (Fig. 2) is satisfactory for this standpoint. It consists of a conventional, amplitude-stabilized, resistance-tuned oscillator, employing 6J7 and 6F6 vacuum tubes, a phase inverter to provide the signal for the last stage, and a push-pull power stage, consisting of two 6V6 vacuum tubes trans-

former-coupled to the bridge. The only important feature of the oscillator is its exceptional amplitude stability, which assures constant amplitude excitation for the bridge. The frequency of oscillation is 1,300 cycles per second.

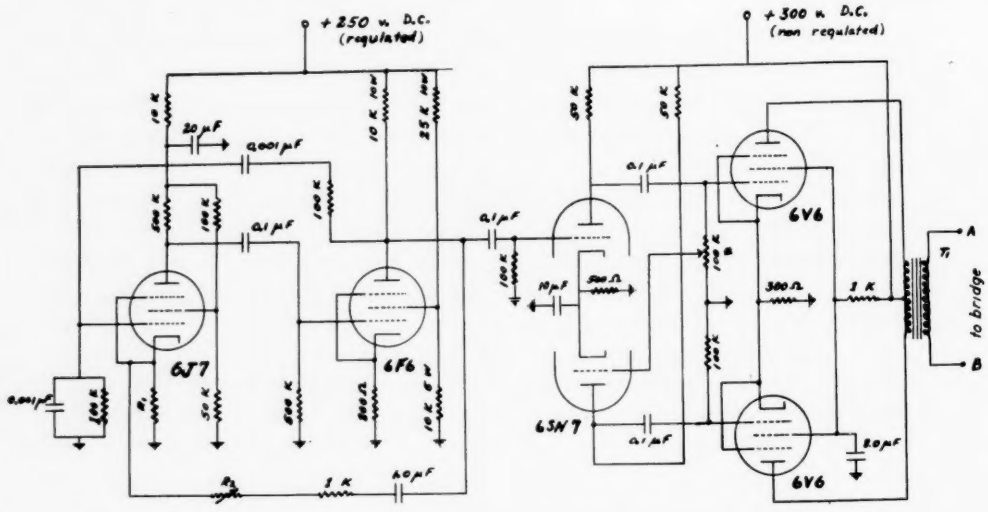

Fig. 2.—The oscillator-power amplifier. The potentiometer marked * should be set so that the A.C. voltages on the plates of the 6SN7 are equal. R_1 is a 6 watt, 110 v. Mazda bulb. R_2 is a 0 to 500 ohm potentiometer which is set to give stable operation of the oscillator. T_1 is selected to match a pair of 6V6's in push-pull (class AB_1) into a 250 ohm load. All filaments are on 6.3 v. A.C. (for discussion see text).

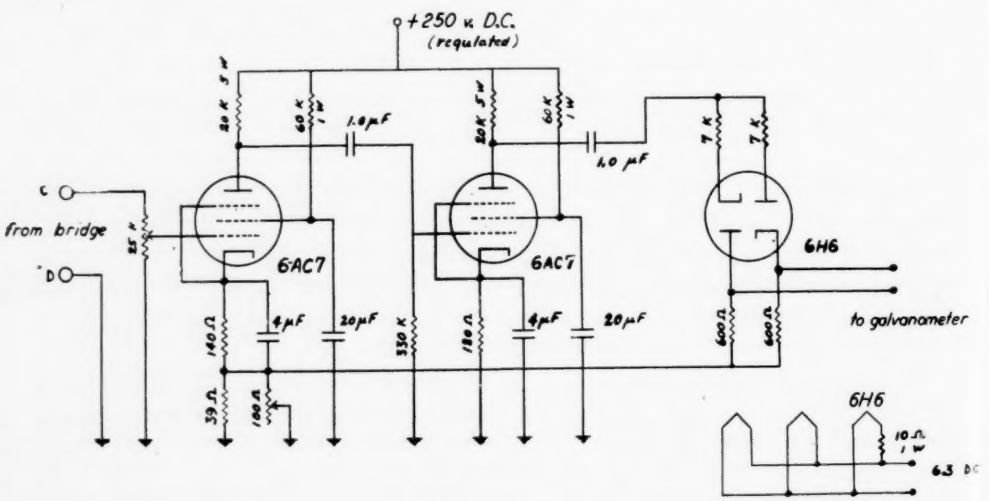

Fig. 3.—The amplifier-detector. Note that all filaments are on 6.3 v. D.C. (for discussion see text).

The technique used in the amplifier-detector has been employed in alternating current vacuum tube voltmeters to overcome nonlinearities of diode detectors. Such detectors usually present a high impedance to signals of small amplitude and low impedance to large signals. This causes a lack of proportionality between carrier amplitude and

detected output. This defect is overcome in the circuit shown (Fig. 3) by including the detecting tube in a feedback loop. Thus, despite changes in the impedance of the diode detector, due to variations in the signal level, the conversion gain of the amplifier detector (in milliamperes through the galvanometer per millivolt of input) is maintained at a practically constant level regardless of the amplitude of the input signal. The degree of linearity between galvanometer current and A.C. input is limited only by the amount of voltage gain in the feedback loop. The conversion gain of this amplifier-detector is about 50 microamperes per millivolt.

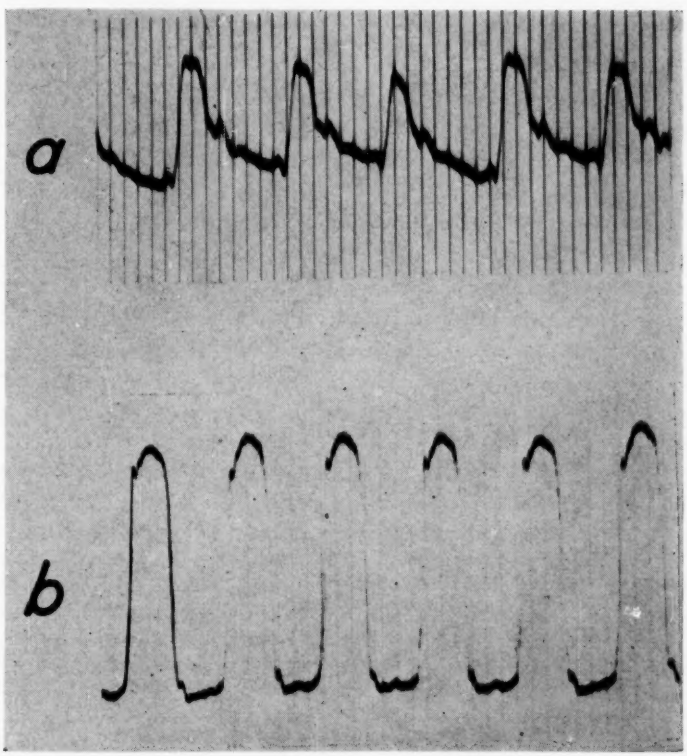

Fig. 4.—Records of (*a*) pulmonary arterial and (*b*) right ventricular pressures obtained using a 100 cm. cardiac catheter. These records were not obtained on the same patient. The first ventricular curve has been retouched in order that the rapid upstroke and downstroke may be seen more easily. The time lines indicate 0.10 second intervals.

This type of detector is not "phase" sensitive; that is to say, either positive or negative pulses of pressure will cause deflections of the galvanometer in the same direction if the transducer bridge is initially balanced to give zero signal output for zero pressure. If, however, the bridge is purposely unbalanced with a trimming resistor, the galvanometer can be made to indicate the sign of the pressure pulse. Thus, instead of having variations of galvanometer current about zero, the variations will be about some value which will serve as zero. Mechanical adjustment of the galvanometer can be used to obtain "on scale" readings.

In operation, the bridge is initially balanced by means of the circuits shown (Fig. 1) to give a zero signal output for zero pressure. Then, by means of the bridge resistance balancing circuit, the bridge is unbalanced in a direction selected to give the proper sign of the pressure pulse as recorded by the galvanometer.

Rapid over-all calibration of the entire system is secured through use of the calibrating resistors (Fig. 1) to produce a change in bridge output which is exactly equal to that produced by a known pressure applied to the transducer.

Some records, typical of those it is possible to obtain with this system, are shown (Fig. 4).

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A MODIFIED PERFUSION TECHNIQUE FOR THE RECOVERY OF SCHISTOSOMES

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INTRODUCTION

IN WORKING on various experiments dealing with schistosomiasis japonica, it was necessary to recover at autopsy all of the parasites regardless of their location or stage of development. The modification of the original Faust and Meleney (1924) perfusion technique by Yolles and co-workers (1947) proved helpful. Further changes have been made in this technique, which are reported here in the hope that they will prove useful to other workers.

PROCEDURE

The following procedure is suggested (Fig. 1).

1. Fill a 1 or 2 gallon aspirating bottle with physiologic saline (0.85 per cent).
2. Use a 100 ml. syringe as a source of pressure.
3. Connect the syringe to the stem of a T tube which is connected to the aspirating bottle (with a stopcock) through one arm and to the needle through the other. Use a 22 gauge needle for mice, and a 19 gauge needle for hamsters.
4. Open the stopcock to fill the syringe and then close it to prevent back flow.
5. Anesthetize the animal with ether, open the abdominal cavity, and ligate the following: (a) inferior vena cava close to the heart, (b) esophagus, (c) the arch of the aorta to the vertebral column, (d) femoral artery, and (e) femoral vein to the femur, (f) sacral artery, and (g) sacral vein to the vertebral column, (h) portal vein, (i) rectum.
6. After the ligations are completed, cut the backbone above the ligature on the aorta and below the sacral artery and then through the leg (including the femur).
7. Separate the backbone and attached organs from the skin and transfer to a Petri dish filled with physiologic saline.
8. Cut the portal vein below the ligature and insert the needle in the aorta, tying it firmly to the backbone so that it will not slip. Then perfuse through the aorta to bring out the worms located in the mesenteric vessels.
9. Now cut the portal vein on the liver side of the ligature and perfuse the liver by inserting the needle in the sinus of the liver.

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REMARKS

The number of Petri dishes used with this technique depends upon the size of the animal, the age of the infection, and the individual worker. The technique described here is excellent for the recovery of immature as well as mature worms. In experienced hands, it requires about one to one and a half hours per mouse or hamster, including the recovery of all of the worms.

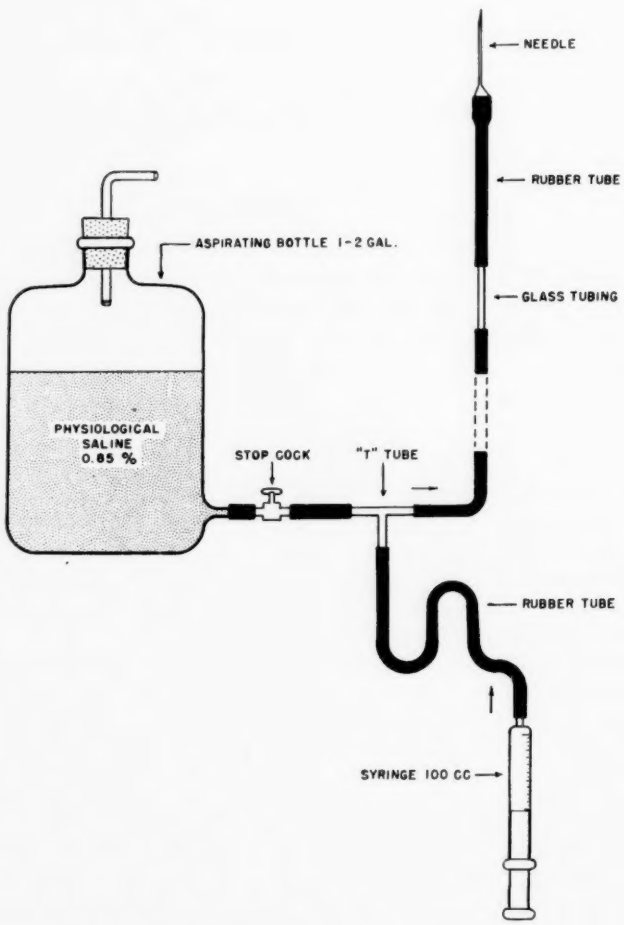

Fig. 1.—Perfusion apparatus for small mammals.

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A TECHNIQUE FOR INFECTING SMALL MAMMALS WITH SCHISTOSOMA JAPONICUM

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INTRODUCTION

A NUMBER of problems have depended on experimental infections of animals with *Schistosoma japonicum*. Over a period of time, an exposure procedure for mice and other small mammals has been developed which gives consistently satisfactory results. In experiments dealing with the effectiveness of a protective ointment, or those concerned with immunologic reactions, it is desirable not only that a high percentage of the cercariae penetrate the skin and reach maturity, but also that the effectiveness of exposure be uniform in individual animals and groups of animals. The technique reported furnishes these advantages.

METHODS

A watch glass, 7.5 cm. in diameter, was filled with paraffin. This was cut away at the center to make a well 6 cm. in diameter. A round hole was then cut in a board approximately 15 cm. square so that the watch glass fitted into the depression. The board with its contained watch glass could then be handled readily on the stage of a dissecting microscope.

Cercariae of *S. japonicum* were obtained by lightly crushing the shell of the snail and removing the liver mass containing the sporocysts and cercariae into a small Stender dish containing dechlorinated tap water. The livers from a number of infected snails were collected, teased apart, and then allowed to remain undisturbed for an hour. Most of the mature or infective cercariae rose to the surface film while the immature ones failed to do so. The mature cercariae were harvested with a capillary pipette and the contents placed as separate and discrete drops in the watch glass. Here the cercariae in each drop were counted before adding additional water.

Animals (mice and hamsters) were shaved down their entire backs, flanks, and necks twenty-four to forty-eight hours prior to exposure. This procedure was critical since contact of the hair with the cercariae resulted in the spreading of the liquid by capillary action, thus entrapping many cercariae in the fur. When ready for use, these small mammals were anesthetized with ether and a small strip of adhesive tape was wrapped around each leg. The animal was wiped with gauze soaked in warm water and placed on its back with the shaven area in contact with the water in the watch glass; it was then immobilized by pinning the free ends of the tape to the board (Fig. 1). Anesthesia was maintained during the first half of the exposure period (approximately fifteen minutes) so that the animal struggled only feebly. In this way it dipped its shaved surface in and out of the water and so provided maximum agitation to the contents of the watch glass. During the last half of the exposure period the animal was permitted to become more active. If necessary, a few drops of water were added from time to time to keep the watch glass from drying out completely. After exposure the mammal was removed to its cage and the watch glass was examined under the microscope to determine the number of cercariae left; these were recorded.

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RESULTS

Thirty mice were each exposed to fifty cercariae of *S. japonicum* by this technique and 857 adult parasites were recovered. The perfusion technique, originally described by Yolles and co-workers (1947) and modified by us (Pan and Hunter) for the recovery of these schistosomes, was followed. An average of 28.6 worms per mouse was recovered; this constituted a 57.1 per cent recovery of the cercariae to which each was exposed. In addition to the mice, ten hamsters were exposed to approximately 200 cercariae each. At autopsy, it was found that these had a total of 1,935 worms or an average of 193.5 worms per animal. The findings also revealed that at least 86 per cent of these were adult worms. Since this was written, other hamsters and mice have been exposed with equally good results.

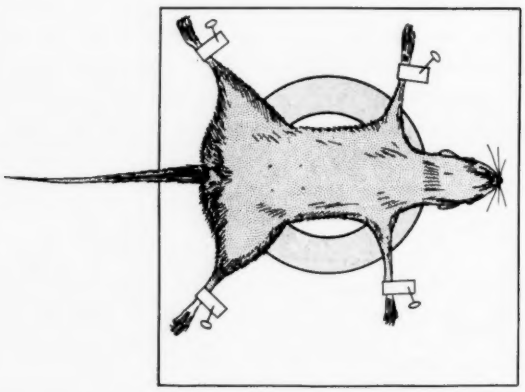

Fig. 1.—Diagram of mouse fastened over watch glass to the animal board.

DISCUSSION

Previously, Cram and Figgat (1947), in working with hamsters, calculated that they recovered as adults only 49 per cent of the cercariae used when the animals were partially immersed, compared with 51 per cent when the cercariae were injected intraperitoneally. This must be compared with our figure of 85 to 95 per cent in hamsters and 57.1 per cent in mice. It should be pointed out that we used a modified Yolles perfusion technique for the recovery of the adults while Cram and Figgat did not. However, while this technique enables one to recover more immature parasites, it has little effect (except in the ease of recovering them) on the total number of adult worms found. Therefore, it is felt that the technique described in this paper will yield a higher percentage of adults than the other techniques now in use for the exposure to cercariae of *S. japonicum*. It is hoped that this technique will prove useful for experimental work using other schistosomes.

SUMMARY

A procedure for infecting small mammals, such as mice and hamsters, is described. This technique necessitates shaving the back, neck, and flanks. Later the mammals are anesthetized and then immobilized over a watch glass containing infective cercariae and exposed for half an hour.

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APPLICATION OF A COLORIMETRIC METHOD FOR ESTIMATION OF METHIONINE IN BLOOD AND URINE

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IN ORDER to study the disappearance of DL-methionine§ after intravenous injections in human beings, a colorimetric method has been modified to permit determination of this amino acid in serum and urine. McCarthy and Sullivan[1] noted that methionine gave a yellow color with alkaline sodium nitroprusside solution, which changed to red on acidification. They applied this reaction to casein hydrolysates. Hess and Sullivan,[2] Csonka and Denton,[3] and Horn, Jones, and Blum[4] modified the McCarthy-Sullivan method and did further work on the methionine content of protein and food hydrolysates. Horn and associates[4] compared the colorimetric procedure with the microbiologic method and obtained similar results with both procedures.

In our preliminary experiments the usual heavy metal protein precipitants for blood and serum, i.e., cadmium hydroxide, zinc hydroxide, and tungstic acid, removed approximately 50 per cent of methionine added to serum. Phosphotungstic acid used by Csonka and Denton to precipitate the basic amino acids from protein hydrolysates likewise removed almost half of the methionine added to serum. When trichloroacetic acid was used as a precipitant for the serum proteins, the loss of methionine increased with its concentration in the serum, reached a maximum (of 20 to 30 mg. per 100 ml.) at about 150 mg. per 100 ml., and then remained constant. It seemed that loss of methionine depended largely on the amount of protein precipitated by trichloroacetic acid.

For a microbiologic determination of L-methionine, Harper, Kinsell, and Barton[5] deproteinized plasma with acetic acid at 100°. This proved a satisfactory method for us insofar as it did not seem to remove any methionine, but the filtrate was cloudy because of incomplete protein precipitation. The experiments with trichloroacetic acid indicated that, if the protein concentration was low, little methionine would be lost. A stepwise precipitation of the protein with acetic acid, heat, and trichloroacetic acid was, therefore, attempted. Recoveries of methionine added to serum were satisfactory (Table I) when carried through this procedure, which is outlined later.

Wheeler and György[6] investigated the applicability of the colorimetric nitroprusside reaction, as described by Csonka and Denton,[3] to normal urine

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§Kindly supplied by Dr. E. F. Roberts, Research Director, Wyeth, Inc., Philadelphia, Pa.

TABLE I. PLASMA METHIONINE RECOVERIES

METHIONINE ADDED (MG./100 ML.)	AVERAGE METHIONINE RECOVERED (MG./100 ML.)	MAXIMUM DEVIATION FROM AVERAGE (MG./100 ML.)	AVERAGE PER CENT RECOVERED
13.3	13.0	0.7	98.0
20.0	19.0	1.0	95.0
33.3	33.2	2.0	100.0
40.0	39.0	1.1	97.5
46.7	47.4	0.7	101.5
66.7	63.8	0.5	96.0
73.3	71.8	2.0	98.0
79.6	81.3	1.6	102.0
100.0	95.9	4.1	96.0
119.4	120.1	0.7	100.5

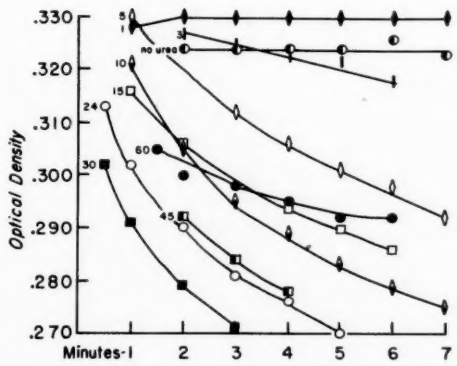

Fig. 1.—Each sample contained 0.4 mg. methionine and milligrams of urea as indicated.

but found that the amounts of methionine present were too small to give accurate and reproducible results. In the present study these low concentrations were not expected, so the colorimetric determination was applied.

In preliminary experiments with urine it was noted that urea caused the methionine color to fade, so that readings made after a few minutes' standing were useless. White and Koch[7] reported a similar phenomenon if cystine was present in high ratios to methionine. They suggested immediate reading after development of the color by addition of acid.

Color development of a mixture of methionine and urea showed that urea and the reagents form a color which absorbs light at the wave length used to determine the concentration of methionine. This urea color is masked by fading of the color due to methionine unless the reading is obtained immediately. Fig. 1 shows how the intensity of the color derived from a constant amount of methionine varies with time and with the amounts of urea present. It may be seen that errors due to the effect of urea may be kept at a minimum if the amount of urea in the aliquot is less than 10 mg. and if the readings are taken within one minute after the addition of acid.

The urea content of each urine sample was determined, therefore, and a dilution made so that the final aliquot did not contain more than 10 mg. of urea. Table II shows methionine recoveries from urine.

TABLE II. URINE METHIONINE RECOVERIES

METHIONINE ADDED (MG./100 ML.)	AVERAGE METHIONINE RECOVERED (MG./100 ML.)	MAXIMUM DEVIATION FROM AVERAGE (MG./100 ML.)	AVERAGE PER CENT RECOVERED
10	14	4	140.0
13	15	2	115.0
20	20	0	100.0
27	26	1	96.0
40	42	2	105.0
60	59	1	98.0
67	66	1	98.5
80	82	2	102.5
100	98	2	98.0
133	123	10	92.5

REAGENTS

The following reagents and standard solutions are required:

(a) 0.4 per cent acetic acid.

(b) 20 per cent trichloroacetic acid.

(c) 15 per cent trichloroacetic acid.

(d) 5N sodium hydroxide (approximately).

(e) 1.3N hydrochloric acid (approximately).

(f) 0.03N hydrochloric acid (approximately).

(g) 1 per cent sodium nitroprusside solution in water, prepared fresh daily.

(h) 85 per cent orthophosphoric acid.

(i) Stock methionine standard: 1.0000 Gm. of DL-methionine is dissolved in 40 ml. approximately 6.4N hydrochloric acid and filled up to 100 ml. with water.

(j) Working methionine standard: the stock methionine standard is diluted 1:100 with water, so that 5 ml. contain 0.5 mg. methionine. If less than 5 ml. are taken to determine the daily calibration factor, 0.03N HCl should be added to bring the volume to 5 ml.

PREPARATION OF FLUIDS

Serum or Plasma.—Into a Pyrex tube calibrated at 7 ml. are placed 3 ml. of plasma or serum and 3 ml. of 0.4 per cent acetic acid. These are mixed well by tapping the tip of the tube. The tube is covered with a marble and placed in boiling water for five minutes to precipitate the proteins. It is then cooled to room temperature and the volume made up to 7 ml. with distilled water. The tube is stoppered, shaken well to break up the protein mass, and centrifuged. Four milliliters of the cloudy supernatant fluid are removed with a pipette, the tip of which is wrapped with a piece of cotton, and placed in a second Pyrex tube. Three milliliters of 15 per cent trichloroacetic acid are added and mixed well by tapping the tube, which is then allowed to stand until the slight precipitate flocculates. If this does not occur at room temperature, the tube may be placed in warm water. The tube is centrifuged, the contents filtered through No. 5 Whatman filter paper until clear, and 5 ml. are taken for color development. A reagent blank and serum blank are set up simultaneously, the first by substituting water for serum and the second on serum taken before the methionine infusion.

Urine.—Approximately 25 ml. of protein-free urine made alkaline to phenolphthalein with 5N sodium hydroxide are refrigerated overnight to precipitate the phosphates. This mixture is filtered while still cold and the urea concentration is determined.

Protein, if present, is removed by adding 2 ml. 20 per cent trichloroacetic acid to 5 ml. of urine. This mixture is allowed to stand for ten minutes, then centrifuged and filtered through No. 5 Whatman filter paper. Five milliliters of the filtrate are placed into a tube calibrated at 7 milliliters. A drop of phenolphthalein is added; the sample is made just alkaline with 5N sodium hydroxide and adjusted to 7 ml. with water. The tube is placed in

an icebox overnight until the phosphates have precipitated and the material is then filtered while still cold. The urea concentration may be determined on the urine before the proteins and phosphates are removed.

Into a 25 ml. volumetric flask is placed an aliquot of the alkaline urine or filtrate containing less than 50 mg. urea so that 5 ml. of the final dilution contain less than 10 mg. of urea (or less than 4.7 mg. urea nitrogen). The sample is made slightly acid by the dropwise addition of approximately 1.3N hydrochloric acid; an additional 0.5 ml. HCl is then added and the mixture is filled up to 25 ml. with water. The color is developed, with the use of 5 ml. of this mixture.

Because of the variable amount of chromogen present, even in a series of urine specimens from a single patient, a urine color blank must be determined for each specimen. This is done by taking 5 ml. of the diluted urine and carrying through the color development except that 1 ml. of water is added instead of the 1 ml. of nitroprusside solution. To correct for the light absorption of the hydrochloric acid, sodium hydroxide, and phosphoric acid mixture, an alkali-acid blank is carried out without nitroprusside on 5 ml. of approximately 0.03N hydrochloric acid.

COLOR DEVELOPMENT

Into a colorimeter tube are placed 5 ml. of the serum filtrate or urine dilution. Standards are run simultaneously because although the calibration curve is a straight line it varies slightly in slope with the room temperature. The useful range on the colorimeter employed for this work was 0.1 mg. to 1.0 mg. of methionine. The reagent blanks are determined by the use of 5 ml. of 0.03N hydrochloric acid instead of serum or urine filtrates.

To the 5 ml. samples are added 2 ml. of approximately 5N sodium hydroxide and 1 ml. of freshly prepared 1 per cent sodium nitroprusside solution. The mixture is allowed to stand exactly ten minutes at room temperature; 2.5 ml. of 85 per cent orthophosphoric acid are added, followed by thorough mixing, and immediately after the disappearance of air bubbles the color intensity is measured (against distilled water) in a photoelectric colorimeter* with a filter with maximum transmission at 515 millimicrons. The methionine concentrations are calculated by correcting for the reagent color and the corresponding blanks.

CALCULATIONS

Let A = Amount of methionine (in milligrams) in standard used for color development,
B = Optical density of the standard,
C = Optical density of the reagent blank containing nitroprusside,

then $\frac{A}{B-C}$ = factor.

For serums

D = Optical density of serum unknown,
E = Optical density of serum blank,
(D–E) × Factor × Dilution $\frac{(49)}{(60)}$ × 100 = Serum methionine milligrams/100 milliliters.

For urines

F = Optical density of urine blank without nitroprusside,
G = Optical density of alkali-acid blank without nitroprusside
H = Optical density of urine unknown
F–G = Urine color blank
Urine color blank + C = Total urine blank
(H–Total urine blank) × Factor × Dilution × 100 = Urine methionine milligrams/100 milliliters.

*A Lumetron colorimeter Model 402E was used.

SUMMARY

A colorimetric method based upon the nitroprusside reaction has been applied to blood and urine. It permits determination of the concentration of methionine to be made with an error of ± 5 per cent in serum and ± 10 per cent in urine.

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A METHOD FOR DETECTING Rh_0 ANTIBODIES IN EXTREMELY LOW TITER

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SINCE the discovery of the Rh factors, a number of methods have been developed for the detection of antibodies specific for these antigens. Saline suspended cells are used for the detection of bivalent antibodies, and cells suspended in protein media[1, 2] as well as the antihuman globulin test (Coombs test)[3] and trypsinated cell[4] method have been used to detect univalent antibodies. These various tests for univalent Rh antibodies differ in their sensitivity, but the trypsinated cell method and the antihuman globulin test are the most delicate and yield the highest titers. Inasmuch as the antihuman globulin test, as originally described, makes use of untreated cells, it was decided to determine the merit of this test using cells which previously had been treated with trypsin. In a recent experiment I used this technique for the first time and found it to be more sensitive than any other.

The purpose of this paper is to report the results of further investigation of this test.

Table I shows the results obtained when nineteen sera containing univalent Rh_0 antibodies were tested by five different titration methods, namely, the saline agglutination, albumin-plasma, trypsinated cell, indirect antihuman globulin, and finally the new method presented in this communication, the trypsinated cell indirect antihuman globulin method. The techniques of the first four of these methods are now well known. The technique of the fifth method is the same as the trypsinated cell method with the exception that after reading the result of that method an antihuman globulin test is done on the cells used with each serum dilution which failed to produce clumping. The titration results for each serum by the five methods employed were obtained by using the same set of cells throughout. Similarly, for the two methods which use trypsinated cells, the same supply of trypsinated cells was used. In this way the titers observed by the various methods can be compared with each other. Examination of Table I reveals that in most cases titration by the trypsinated cell indirect antihuman globulin test yielded the highest figures, although in some instances it differed little or not at all from readings obtained by the indirect antihuman globulin method. However, in two cases antibodies were detectable only by this method, whereas the results with the other four were completely negative. The average titer by the trypsinated cell indirect antihuman globulin method was slightly more than twice that of the same test carried out with untreated cells. The test would hardly be worth the trouble if all that one obtained was a titer approximately double that of the indirect antihuman globulin method. It is felt that its value rests in its ability (1) to

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TABLE I. TITRATION RESULTS OBTAINED BY VARIOUS METHODS

SERUM	METHODS AND TITERS (UNITS)				
	SALINE	ALBUMIN-PLASMA	TRYPSINATED CELL	INDIRECT ANTI-HUMAN GLOBULIN UNTREATED CELLS	TRYPSINATED CELL INDIRECT ANTI-HUMAN GLOBULIN
1	0	8	23	64	200
2	0	0	1	4	7
3	0	12	14	96	109
4	0	6	4	50	40
5	0	10	16	40	134
6	0	0	0	0	8
7	0	0	2	3	7
8	0	0	1	1	16
9	0	0	2	2	6
10	0	0	5	1	10
11	0	1	6	7	20
12	0	4	11	8	14
13	0	6	11	16	64
14	0	1	2	4	6
15	0	3	3	16	64
16	0	1	8	16	32
17	0	0	0	0	2
18	0	0	2	0	4
19	0	4	16	24	64

detect Rh_0 antibodies present in such low concentration that they cannot be measured by other methods and thereby aid in the diagnosis of Rh sensitization, and (2) to identify antibodies hitherto undescribed. To test the first premise further, a serum known to contain univalent Rh_0 antibodies in a titer of 256 units was diluted as follows: 1:500, 1:750, 1:1,000, 1:1,500 (Table II). These dilutions were tested against group O type Rh_1, group O type Rh_2, and group O type rh cells using the saline agglutination, albumin-plasma, trypsinated cell, indirect antihuman globulin, and the trypsinated cell indirect antihuman globulin methods. Whereas all dilutions were negative by the saline agglutination and the albumin-plasma methods, both the trypsinated cell and the indirect antihuman globulin methods showed one plus clumping only in the 1:500 dilution. Nevertheless, the more sensitive trypsinated cell

TABLE II. TITRATION RESULTS FOR Rh_0 UNIVALENT ANTIBODIES IN EXTREMELY LOW TITER

METHOD	CELLS	DILUTION OF SERUM*				TITER (UNITS)
		1:500	1:750	1:1,000	1:1,500	
Saline agglutination	O Rh_1	–	–	–	–	
	O Rh_2	–	–	–	–	0
	O rh	–	–	–	–	
Albumin-plasma	O Rh_1	–	–	–	–	
	O Rh_2	–	–	–	–	0
	O rh	–	–	–	–	
Trypsinated cell	O Rh_1	+	–	–	–	
	O Rh_2	+	–	–	–	500
	O rh	–	–	–	–	
Indirect antihuman globulin	O Rh_1	+	–	–	–	
	O Rh_2	+	–	–	–	500
	O rh	–	–	–	–	
Trypsinated cell indirect antihuman globulin	O Rh_1	++	++	+±	tr.	
	O Rh_2	++	++	+±	+	1,250
	O rh	–	–			

*Titer of the original undiluted serum was 256 units by the albumin-plasma method.

indirect antihuman globulin method resulted in two plus clumping in the 1:500 and 1:750, one and one-half plus in 1:1,000, and weak clumping in the 1:1,500. The titer by this method, therefore, was not only higher but the reactions were stronger than those obtained by the others.

Inasmuch as the titer for univalent Rh_0 antibodies of the undiluted serum used for this experiment was 256 units, the titer of the 1:1,000 dilution was approximately one-quarter of a unit, which is below the range of the less sensitive methods. If a patient's serum actually contained antibodies in a concentration identical with that of our artificial preparation, the diagnosis of sensitization would have been impossible with all tests except the trypsinated cell indirect antihuman globulin test.

SUMMARY

A highly sensitive test for titration of univalent Rh_0 antibodies is described. This technique yields titers approximately double those obtained by other methods. The method is of value to detect antibodies present in such low concentration that they are below the threshold of other methods. It is possible that this method may prove of value in identifying new antibodies.

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AN INTEGRATING VENTILATION METER FOR CLINICAL USE

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THE measurement of lung ventilation rates in patients ordinarily requires relatively complicated procedures or expensive and cumbersome apparatus. Furthermore, in most instances the ventilation rate must be calculated from data obtained by the measuring device. Physicians have, therefore, usually relied on visual observation to judge the adequacy of a patient's respiratory minute volume. Such observations are probably not more reliable than the clinical estimation of cyanosis, which has been shown to be a conspicuously misleading guide to the degree of arterial oxygen saturation.[1] For example, poliomyelitis patients being treated in the body respirator may be seriously hyperventilated.[2] On the other hand, severe underventilation frequently occurs during surgery, because of the inability of anesthetists to estimate a patient's ventilation by watching the breathing bag on the anesthesia machine.[3] Hyperventilation and hypoventilation disturb acid-base balance, may cause circulatory and renal complications, and alter the sensitivity of the respiratory center to carbon dioxide.

A simple, inexpensive minute volume indicator has been devised, using a modified venturi tube with pressure taps arranged according to the method of Goldman.[4] As the patient breathes in either direction through a tube (Fig. 1), static energy is converted to kinetic energy as the air velocity increases in the narrow region of the tube. The center tap (A) therefore measures a static pressure which is always negative relative to the pressure at the ends of the tube (B and B'). The difference in pressure between A and BB' is a function of air flow. By connecting A and BB' to the two sides of a differential draft gauge,* a continuous indication of air-flow velocity may be obtained. The system must be further damped by capillary tubes inserted at C and D. When suitable damping has been obtained, the draft gauge indicator will remain almost stationary and at any instant will indicate the mean air-flow rate present during the preceding several breaths. The instrument can be calibrated in terms of liters per minute ventilation, and since the mechanical damping essentially integrates the flow rates, it is necessary only to glance at the indicator to know how much the patient will have breathed at the end of a minute if he continues to breathe at that rate.

Calibration was done with a Benedict-Roth type of recording spirometer using mechanical pumps and a series of individuals breathing in a variety of flow patterns at rates ranging from 11 to 36 per minute. Fig. 2 shows the calibration curve obtained. The average deviation of the measurements at resting ventilation rates (6 to 10 liters per minute) was found to be ±2.65 per cent. Three values out of twenty-six observations in this range had an error of more than 5 per cent, the largest being 8.9 per cent. Since the gauge is differential, it may be used with positive pressure breathing appliances as well as with normal

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*A Magnehelic gauge made by F. W. Dwyer Co., Chicago, Ill., was used. An inexpensive, inclined liquid manometer has been found to work equally well.

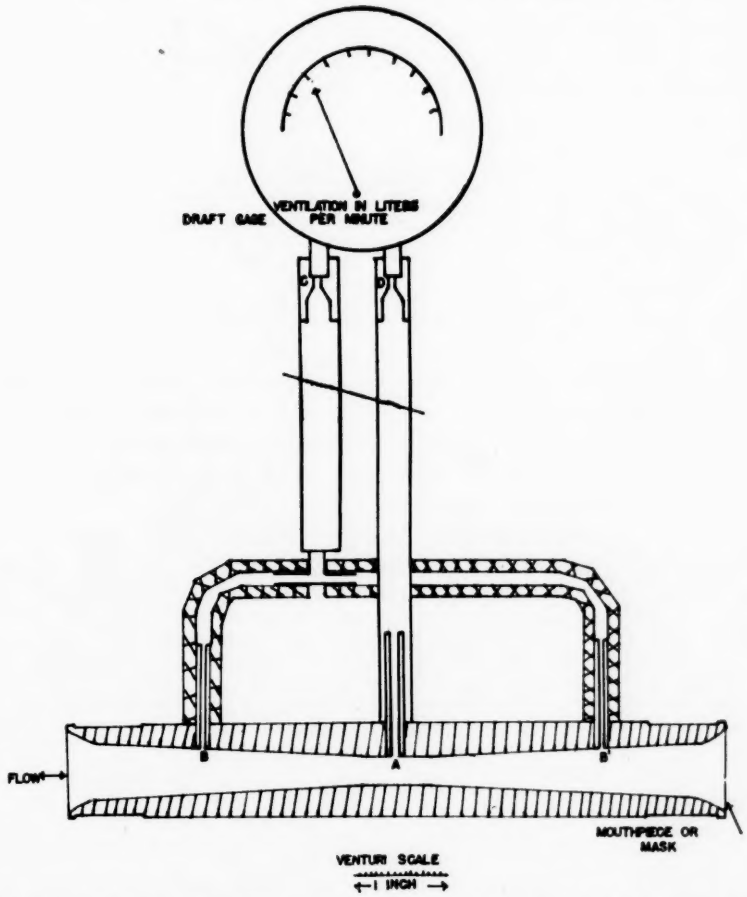

Fig. 1.—Diagram of ventilation meter assembly. *A*, Venturi side pressure tap; *B*, *B'*, reference pressure taps; *C* and *D*, capillary constrictions at connections to differential pressure gauge.

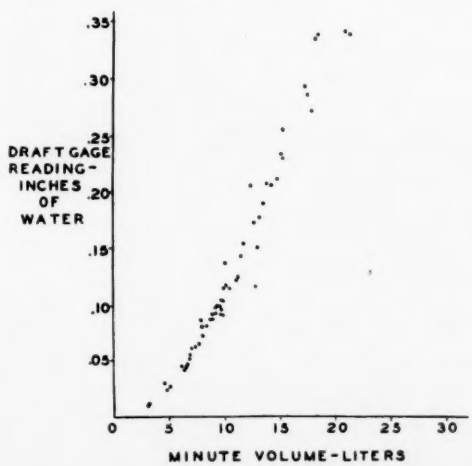

Fig. 2.—Calibration data for ventilation meter. Minute volume measured by spirometer. Points represent gauge readings over a wide range of respiration rates and air-flow patterns.

breathing. Because of the low energy loss feature of the venturi, the tube offers very little resistance to air flow (3.7 mm. H_2O at the normal peak flow rate of 34 liters per minute). Resistances found at the normally occurring flow rates were:

FLOW (liters/min.)	RESISTANCE (mm. H_2O)
10	0.7
20	1.7
30	3.0
40	5.0

The use of the venturi principle minimizes resistance, expense, and "coasting" error met with in our attempts to use rotary anemometers for the same purpose.

In clinical use, the ventilation meter has proved valuable in adjusting the ventilation given to poliomyelitis patients being treated in the body respirator. Incorporation of the instrument into standard anesthesia machines provides the anesthetist with a continuous indicator of respiratory minute volume as a guide to when patients need respiratory assistance.

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ERRATUM

In the article by Gaberman, Atlas, Isaacs, Kammerling, and Ehrlich, "The Relationship of Xanthoproteic Indices to Quantitative Phenol Levels in Various Azotemic States," p. 544, in the April, 1951, issue of the JOURNAL, all parentheses should be removed from all formulas. The formulas thus will read as follows:

Phenol = .0319 Xanthop. − .035
Xanthop. index = 24.95 Phenol + 17.9
Approximate combined equation: Xanthoproteic index = 27.8 Phenol + 10